IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
GREENFIELD et al.
Serial No. 09/155076
Filed: March 21, 1997
For: "PEPTIDE FROM SOLUBLE FORM OF ACETYLCHOLINESTERASE, ACTIVE AS A CALCIUM CHANNEL MODULATOR"

DECLARATION

The Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, Martin Westwell, do hereby declare and state as follows:

1. I am a British citizen of 13 Mill Road, Abingdon, Oxfordshire OX14 5NS. I currently hold the position of Research Manager at Synaptica Limited and until 30 June 2001 I was the Research Coordinator of Synaptica Limited and held a fellowship at Lincoln College, Oxford University in biological/medicinal sciences. Synaptica Limited is the assignee of US Patent Application serial no. 09/155076.

2. I have read the comments of the Examiner in the Office Communication dated 22nd June 2001 concerning the above-identified US Patent Application. As a result of my position in Synaptica Limited, I have first hand-knowledge of technical data relevant to the suggestions by the Examiner that whole human acetylcholinesterase (AChE) and the 40 amino acid residue C-terminal fragment of human AChE (the product of exon 6 of the AChE gene) share the calcium channel modulatory function of Synaptica

peptide (the 14mer of SEQ. ID no. 1 as noted in US Patent Application Serial no. 09/155076). These suggestions of the Examiner are wrong as evident from technical data discussed below.

3. Synaptica peptide is contained within the C-terminal 40 mer tail sequence of human AChE (commonly referred to as the T40 peptide) but has both distinct structural and functional characteristics from that longer peptide. In particular, at nanomolar concentrations, Synaptica peptide has been shown to allosterically potentiate the response of the alpha 7 nicotinic receptor to acetylcholine and other agonists of that receptor. The T 40 peptide does not exhibit this modulatory function as shown by the data in Figure 1 below. The data in Figure 1 was obtained using the biotinylated and amidated version of Synaptica peptide. However, additional experiments documented in Published International Application WO 01/73446 of Synaptica Limited (a copy of which is annexed hereto as Exhibit 1) provide further evidence that Synaptica peptide is capable of modulating calcium flux through alpha 7 nicotinic receptors. It will be noted that I am a named inventor on that patent application.

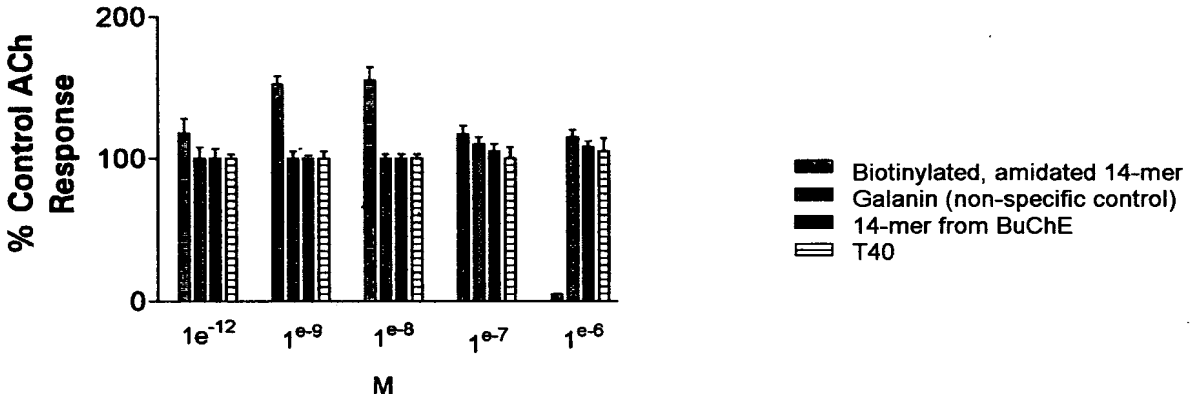


Figure 1 *The positive allosteric effect of the biotinylated and amidated 14-mer can be seen at nanomolar concentrations. The experiments were carried out using Xenopus oocytes injected with α7 nAChR RNA. Impaled oocytes were superfused with an EC50 concentration of acetylcholine (100 μM) and then 100 μM acetylcholine plus increasing concentrations of the peptides under study. Data represents the mean ± SEM of 4 independent experiments (4 different batches of oocytes)*

In keeping with the above-noted different functional activity of T40 peptide and Synaptica peptide, circular dichroism spectroscopy shows that an α-helical structure

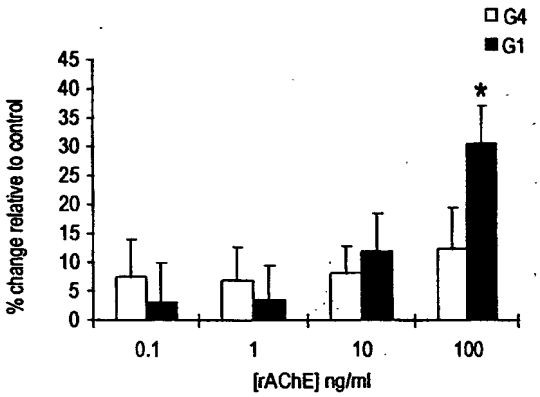
dominates the T40 peptide whereas Synaptica peptide is random coil, β-turns or β-sheet depending upon the conditions.

4. The Examiner is correct that Example 3 of US Patent Application Serial no. 09/155076 suggests that whole AChE causes calcium influx into neurons. However, further experiments such as those described above in Figure 1 indicate that this does not reflect that AChE shares the same non-enzymic mechanism of action as Synaptica peptide. The AChE protein is not a functional analogue of Synaptica peptide. This conclusion is consistent with experiments looking at the ability of G_1 AChE (the recombinant monomeric form of terameric AChE minus the T40 tail) and Synaptica peptide to influence neurite outgrowth of cultured neuronal cells. On cultured hippocampal cells, Synaptica peptide at 1 to 10 nM causes a brief period of neurite outgrowth prior to apoptosis (cell death). Increasing the concentration and/or incubation time of the 14 mer causes a clear apoptotic-necrotic continuum (see Table 1 below). This can be explained in terms of change of calcium flux into the neurons. In contrast, full length G_1 AChE (3 U/ml) causes a robust neurotrophic response (see Figure 2 below) consistent with a different non-cholinergic action from Synaptica peptide. In the same system, T40 peptide has no response on cell survival and/or health.

Incubation time (hours)	[Synaptica peptide]	Mode of cell death
1	1 – 10 nM	Compensatory
24	1 nM – 1 mM	No effect
48	100 nM – 1 μM	Apoptosis
72	10 nM – 1 μM	Apoptosis
72	10 μM – 1 mM	Necrosis
336	10 μM – 1 mM	Necrosis
336	1 nM – 1 μM	Apoptosis

Table 1 *The action of the 14-mer peptide on cultured hippocampal neurons is dependent on dose and incubation time.*

Figure 2: the non-cholinergic ability of AChE to enhance neurite outgrowth of cultured neurons. Organotypic neurons of the substantia nigra were used to show the neurotrophic effect of monomeric AChE. This effect is significant at 100ng/ml.



5. The conclusion must be that processing of whole AChE or the T40 peptide is required to produce the biological activity exhibited by Synaptica peptide. In support of such processing underlying linkage of AChE to neurodegenerative disease causation, it has been shown that Synaptica peptide can be injected into the brains of rats to cause attentional deficit reminiscent of Alzheimer's disease. Such studies are documented in Published International Application no. WO 01/49107, also in the name of Synaptica Limited, a copy of which is annexed as Exhibit 2.

6. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of this declaration, the patent application, or any patents issuing thereon.

Martin Westwell

Martin Westwell

18 October 2001

Date

EXHIBIT 1

International Application WO 01/73446

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number
WO 01/73446 A1

(51) International Patent Classification[7]: G01N 33/94, C12Q 1/46

(21) International Application Number: PCT/GB01/01401

(22) International Filing Date: 29 March 2001 (29.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0007630.7 29 March 2000 (29.03.2000) GB
0030660.5 15 December 2000 (15.12.2000) GB

(71) Applicant *(for all designated States except US)*: **SYNAPTICA LIMITED** [GB/GB]; 551 Harwell, Harwell International Business Centre, Didcot, Oxon OX11 0QJ (GB).

(71) Applicant *(for US only)*: **GREENFIELD, Susan, Adele** [GB/GB]; University Department of Pharmacology, Mansfield Road, Oxford OX1 0QT (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **WESTWELL, Martin** [GB/GB]; Lincoln College, Oxford OX1 3DR (GB).

(74) Agents: **IRVINE, Jonquil, Claire** et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/73446 A1

(54) Title: ALPHA 7 NICOTINIC RECEPTOR SCREENING ASSAYS

(57) Abstract: The present invention relates to screening assays for functional analogues and antagonists of a polypeptide fragment of acetylcholinesterase (AChE), which is believed to exhibit activity corresponding to non-enzymatic function of AchE in the brain. These assays stem from linkage of such non-enzymatic activity with a target site on the homomeric alpha 7 nicotinic receptor. Antagonists thus identified which are capable of formulation for passage through the blood-brain barrier may be advantageous therapeutic agents for the treatment of a number of neurodegenerative diseases, in particular Alzheimer's Disease, Parkinson's Disease and Motor Neuron Disease.

ALPHA 7 NICOTINIC RECEPTOR SCREENING ASSAYS

The present invention relates to screening assays for selecting agonists and antagonists for non-enzymatic biological activity of acetylcholinesterase (AChE), which is believed to be mediated in the brain by a polypeptide fragment of the enzyme. In particular, it relates to such assays stemming from identifying the receptor site for a 14 mer fragment of AChE (SEQ. ID. No. 1) as a modulatory site present on the homomeric alpha 7 nicotinic receptor. Antagonists thus identified which are capable of formulation for passage through the blood-brain barrier are envisaged as therapeutic agents for the treatment of a number of neurodegenerative diseases, in particular, for example, Alzheimer's Disease, Parkinson's Disease and Motor Neuron Disease.

Background to the invention

It has for some years been proposed that AChE has a non-enzymatic action in the brain, which appears to share close parallels with the action of amyloid precursor protein (APP). Evidence has previously been presented indicating that this non-enzymatic action of AChE underlies a trophic function in developing brains but if activated in adult brains leads to neurodegenerative disorders. This hypothesis for neurodegenerative disease causation is currently of particular interest in relation to, for example, Alzheimer's Disease, Parkinson's Disease and Motor Neuron Disease.

Although Alzheimer's Disease and Parkinson's Disease have different clinical profiles, it has long been acknowledged that the underlying pathologies can overlap. Causation of both diseases can be attributed to different degrees of disruption to neuronal groups within the globally-projecting neurons extending from the spinal cord to midbrain (referred to as the "isodendritic core"; Rosser, British Medical Journal, (1981) 283, 1588-1598). Studies of this group of neurons have revealed important differences from the rest of the brain (Woolf, Neuroscience (1996) 74, 625-651). Importantly, they have been found to retain the capacity in adult brain for

not only axonal regeneration, but also for proliferation. It has previously been suggested that vulnerability of global neurons to degeneration might be associated with aberrant activation of a developmental mechanism in response to local insult. More recently, evidence has been presented leading to linkage of this hypothesis to proposed trophic function of AChE in developing brains mediated by promotion of Ca^{2+} entry into immature neurons (Greenfield, Spring Research News (1997) 2-3; Greenfield, Brit. Med. J. (1998) 317, 19-26; Webb et al., Eur. J. Neurosci.(1996) 8, 837-841; Holmes et al., J. Neuro. Res. (1997) 49,1-12.). Significantly, although sub-populations of global neurons contain different neurotransmitters, irrespective of whether they are cholinergic neurons acted upon by acetylcholine, they contain AChE (Greenfield, Neurochem. Int.,1996). Furthermore, it is recognised that immature neurons can withstand higher levels of intracellular calcium than their mature counterparts (Eimerl and Schramm, J. Neurochem. (1994) 62,1223-1226). Indeed, it has been shown that an amount of calcium that will be beneficial in developing neurons will kill their mature counterparts.

The global neuronal population of cells contain not only neurons associated with Alzheimer's Disease and Parkinson's Disease, but also neurons associated with Motor Neuron Disease. Motor neurons have been shown to release AChE (Rodriguez et al., J. Neurol. Sci. (1997) 152, Suppl. 1: S54-61) and embryonic spinal rat motor neurons have also been reported to be sensitive to its trophic action in culture (Kreutzberg et al., Advances in Neurol. (1974) 12, 269-281; Brimjoin, Prog. Neurobiol. (1983) 21, 291-322; Moreno et al., Brain Research (1996) 718, 13-24; Bataillé et al., Eur. J. Neurosci. (1998) 10 (2) 560-572). Hence, there is reason to believe that Alzheimer's Disease, Parkinson's Disease and Motor Neuron Disease have a single underlying causality related to non-enzymic function of AChE.

Published International Application WO 97/35962 discloses a 14 mer biologically active fragment of AChE (Synaptica Peptide) having the sequence AEFHRWSSYMVHWK (SEQ. ID. No. 1), which corresponds to amino acid

residues 535 to 548 of the mature protein. Evidence is presented in the same International Application that Synaptica Peptide represents a portion of AChE retaining its capacity to mediate non-enzymatic biological effects. An *in vivo* counterpart is known to be cleaved from AChE, but the exact nature of the polypeptide fragment which is ultimately responsible in the brain for mediating non-enzymatic activity of AChE remains to be elucidated.

As shown in Figure 1 of WO 97/35962, and Figure 1 of the present specification, the sequence of Synaptica Peptide is conserved between AChE of different species, including human and rat AChE, and exhibits similarity to a region of human APP (the region at the N-terminus of the Aβ1-42 fragment which has been associated with Alzheimer's Disease). However, the same sequence or a closely similar sequence has not been found in any butyrylcholinesterase, which like AChE hydrolyses acetylcholine. As also reported in WO 97/35962, electrophysiological studies with slices of adult guinea pig midbrain showed that Synaptica Peptide initially enhances calcium potentials in neurons of the substantia nigra induced either by N-methyl-D-aspartate (NMDA) or by direct depolarisation of neurons. With sustained application of Synaptica Peptide for a few minutes, however, a reduction in calcium entry is observed. This is in keeping with so much calcium entering the neurons that calcium channels are shut-off. The same modulation of calcium influx is not observed with a comparable peptide fragment from butyrylcholinesterase or Aβ1-42.

It has since been shown that Synaptica Peptide can also enhance Ca^{2+} flux into hippocampal neurons which eventually switches off calcium channels. This is of particular interest in relation to Alzheimer's Disease since the hippocampus is the major site of degeneration neuropathology associated with that disease.

On the basis of further studies with Synaptica Peptide, and a biotinylated and amidated version of the same peptide, the target receptor site for Synaptica Peptide is now proposed to be an allosteric modulatory site present on the homomeric alpha 7 nicotinic

receptor. This receptor has previously been identified as a nicotinic receptor having high calcium permeability, which is blocked by α-bungarotoxin. By transfecting *Xenopus* oocytes with mRNA for rat alpha 7 subunits, functional homomeric alpha 7 nicotinic receptors having 5 alpha 7 subunits have previously been obtained for study *in vitro* (Séquéla et al., J. Neurosci. (1993) 13, 596-604). Published International Application WO 94/20617 also describes cloning of the cDNA for the human alpha 7 nicotinic receptor subunit and engineering of human cells and *Xenopus* oocytes to express functional homomeric alpha 7 nicotinic receptors. It is believed that such receptors mimic homomeric alpha 7 nicotinic receptors *in vivo* (Chen and Patrick, J. Biol. Chem. (1997) 272, 24024-24029; Drisdel and Green, J. Neurosci.(2000) 20,133-139). Such receptors appear transiently in developing brain and occur in regions where AChE is believed to have non-enzymatic function (Broide et al., Neurosci. (1995) 67, 83-94; Broide et al., J. Neurosci. (1996) 16, 2956-2971; Kim et al., Develop. Brain Res. (1995) 85, 283-287; Tengelsen et al., Brain Res. (1992) 594, 10-18). Alpha 7-subunit containing nicotinic receptors, which are sensitive to α-bungarotoxin, have also been found in adrenomedullary chromaffin cells (Lopez et al., Proc. Natl. Acad. Sci. USA (1998) 95, 14184-14189; Criado et al., J. Neurosci. (1997) 17, 6554-6664). Importantly, it has also been reported that alpha 7 subunit mRNA can be found in post-mortem brain tissue from the hippocampus of Alzheimer's Disease patients (Hellström-Lindhal et al., Mol. Brain Res. (1999) 66, 94-103).

That alpha 7 nicotinic receptors are subject to allosteric modulation via binding of ligands at a site distinct from the site for binding of ion-channel agonists has previously also been shown. For example, ivermectin, in the μM range, has been found to strongly enhance acetylcholine-evoked currents through neuronal chick and human homomeric alpha 7 nicotinic receptors in oocytes (Krause et al., Mol. Pharm. (1998) 53, 283-294). The allosteric site targeted by ivermectin in such studies is believed to be distinct from the binding site on alpha 7 nicotinic receptors for Aβ1-42. Binding of Aβ1-42 to such receptors has been reported, for example, in Published International Application no. WO 99/62505, but, as indicated above, Aβ1-42 has not been found to have a modulatory effect on induced Ca^{2+} flux. Previously it has been proposed that potentiators capable of acting at the ivermectin site to increase Ca^{2+} flux could be of interest to compensate for

the deleterious effects of neurodegenerative disorders. The additional linkage of non-enzymatic function of AChE with an allosteric modulatory site on brain alpha 7 nicotinic receptors importantly now provides for the first time impetus for identification of antagonists of modulatory ligands on such receptors, particularly Synaptica Peptide, in relation to devising new treatments for Alzheimer's Disease and other neurological disorders aimed at preventing, or at least inhibiting, neuronal deterioration.

Summary of the invention

The finding that Synaptica Peptide targets a modulatory site on brain alpha 7 nicotinic receptors provides the foundation for new assays for screening for both functional analogues and antagonists of that peptide.

Thus, in one aspect, the present invention provides use of an alpha 7 nicotinic receptor, preferably a human alpha 7 nicotinic receptor, or a functional analogue thereof, to determine whether a compound is capable of acting as a functional analogue or antagonist of the polypeptide of SEQ. ID. No. 1 on said receptor. Where a native alpha 7 nicotinic receptor is employed in its normal membrane environment, it will be identified by means of inhibition by an alpha 7 nicotinic receptor blocker such as α-bungarotoxin. By functional analogue in this context will be understood a variant of a native alpha 7 nicotinic receptor which retains a modulatory binding site for Synaptica Peptide and ability to exhibit induced Ca^{2+} permeability which is influenced by binding of Synaptica Peptide to the same variant.

In one embodiment, there is thus now provided a method for determining the ability of a compound to act as an antagonist of the polypeptide of SEQ. ID. No. 1 (Synaptica Peptide), which comprises determining whether said compound can inhibit binding of Synaptica Peptide or a functional analogue thereof to its target site on an alpha 7 nicotinic receptor or functional analogue of said receptor and thereby antagonise the modulatory effect of Synaptica Peptide or its analogue on induced ion flux, e.g. Ca^{2+}

flux, through the receptor. It will be appreciated that such a method may be applied to screen compounds as part of a screening programme aimed at identifying compounds for use in preventing, inhibiting or reversing neurological disorders believed to be associated with non-enzymatic function of AChE, especially Alzheimer's Disease, Parkinson's Disease and Motor Neuron Disease.

In such a method, Synaptica Peptide may be employed or any compound which is capable of mimicking the modulatory effect of Synaptica peptide at its modulatory binding site on an alpha 7 nicotinic receptor. It will be appreciated that such functional analogues of Synaptica Peptide include, but are not limited to, variants of Synaptica Peptide having one or more additions and/or deletions and/or substitutions, e.g. conservative substitutions compared to SEQ. ID. No. 1 which result in retention of its calcium channel modulatory function. Such an analogue may be, for example, Synaptica Peptide with an N-terminal and/or C-terminal extension. It may have at least 80%, at least 90%, at least 95% or more, e.g. 99% homology or identity with the sequence of SEQ. ID. No. 1 over its entire length or a portion of its length. It may be the *in vivo* counterpart of Synaptica Peptide.

The term "alpha 7 nicotinic receptor" will be understood to refer to a homomeric receptor of alpha 7 subunits which exhibits in the presence of Ca^{2+} ions and acetylcholine (ACh) induced Ca^{2+}flux which can be (i) blocked by α-bungarotoxin and (ii) modulated by Synaptica Peptide. Such a receptor may be a native homomeric alpha 7 nicotinic receptor in its normal membrane environment or a homomeric alpha 7 nicotinic receptor inserted into a synthetic membrane or a cellular membrane which does not normally present alpha 7 nicotinic receptors.

A method of the invention as set out above may additionally include the step of determining whether a compound proposed for test, or selected as an agonist or antagonist, is capable of crossing the blood-brain barrier or of formulation for passage across the blood-brain barrier. Antagonists identified by a method as set out above and

which are able to pass through the blood-brain barrier are also expected to be capable of antagonising non-enzymatic function of AChE in the brain and hence to be potentially useful therapeutic agents for treatment of neurodegenerative disorders including, for example, as previously indicated above Alzheimer's Disease and Parkinson's Disease.

Brief description of the figures

Figure 1: Alignment of 5 partial AChE sequences including the Synaptica Peptide sequence, the equivalent regions of 3 butyrylcholinesterases (BChE sequences) and a portion of the human A4 amyloid precursor protein (Hum Amyl). Hum AChE = human AChE; Rab AChE = rabbit AChE; Mus AChE = mouse AChe; Bov AChE = bovine AChE; Hum BChE = human BChE; Rab BChE = rabbit BChE; Mus BChE = mouse BuChE. Residues in bold are conserved across all sequences. Boxed residues are shared by all AChEs and human APP, but by no BChE. The bar above the alignment shows the position of the Synaptica Peptide sequence. The bar below the alignment indicates the homologous region of human APP at the N-terminus of the Aβ1-42 fragment.

Figure 2: Proposed sequence of events whereby activation of non-enzymatic function of AcheE leads to neurodegeneration in the global neuron population.

Figure 3: Current-voltage relationship for the human alpha 7 nicotinic receptor presented at the surface of *Xenopus* oocytes;

Figure 4: Concentration-response relationship for ACh on human alpha 7 nicotinic receptors presented at the surface of *Xenopus* oocytes.

Figure 5: Figure illustrating enhancement by Synaptica Peptide of the ACh-induced Ca^{2+} flux through human alpha 7 nicotinic receptors expressed by *Xenopus* oocytes when there is no pre-incubation with the peptide.

Figure 6: Figure illustrating inhibition of the ACh induced Ca^{2+} flux through human alpha 7 nicotinic receptors expressed by *Xenopus* oocytes when the oocytes are pre-incubated with the peptide for 2 minutes.

Figure 7: Figure showing the effect of different concentrations of Synaptica Peptide on ACh currents through human alpha 7 nicotinic receptors expressed by *Xenopus* oocytes when the oocytes are either not pre-incubated with the peptide or incubated with the peptide for 2 minutes.

Figure 8: Intracellular recordings in *Xenopus* oocytes expressing human alpha 7 nicotinic receptors in the presence of (i)100 μM ACh (ii) 10 μM Synaptica Peptide and (iii) 10 μM Synaptica Peptide when followed by addition of 100 μM ACh after 30 seconds.

Figure 9 shows dose-dependent decrease in ^{125}I-α-bungarotoxin binding to human SH-SY5Y neuroblastoma cells in the presence of biotinylated and amidated Synaptica Peptide.

Figure 10 illustrates decrease of viability of human SH-SY5Y neuroblastoma cells in the presence of biotinylated and amidated Synaptica Peptide.

Detailed description of the invention

It will be appreciated that screening assays according to the invention may follow a number of protocols. Native alpha 7 nicotinic receptors may be employed without isolation from their normal membrane surrounding, e.g. in the form of a brain tissue slice maintained *in vitro*, an organotypic tissue culture comprising neuronal cells such as neonate hippocampal cells or cultured cells. Suitable cultured cells for this purpose include, for example, cultured PC-12 cells (rat pheochromocytoma cells) which have previously been shown to express functional α-bungarotoxin-sensitive alpha 7 nicotinic

receptors composed of homomers of alpha 7 subunits (Blumenthal et al., J. Neurosci. (1997) 17, 6094-6104, Rangwala et al., J. Neurosci. (1997) 17, 8201-8212 and Drisdel and Green, J. Neurosci. (2000) 20, 133-139). Alternatively, for example, cultured human SH-SY5Y neuroblastoma cells may be employed either expressing their normal level of autologous alpha 7 nicotinic receptor or after transformation to increase alpha 7 nicotinic receptor expression, e.g. by providing additional expression of a heterologous alpha 7 nicotinic receptor as described in Puchacz et al., FEBS Let. (1994) 354, 155-159. Where native alpha 7 nicotinic receptors are employed without isolation from their normal membrane environment, binding of Synaptica Peptide or the test compound to such receptors and/or change of ion flux as a result of such binding will be shown by use of an alpha 7 nicotinic receptor blocker, preferably α-bungarotoxin or a suitable antibody.

Alternatively, however, and preferably alpha 7 nicotinic receptors or functional analogues thereof may be employed inserted into a synthetic membrane or presented at the surface of cells or membrane preparations derived therefrom which do not normally present alpha 7 nicotinic receptors. Such cells will preferably be derived from cells which do not normally express any Ca^{2+} permeable receptor. They may, for example, be human cells, e.g. human embryonic kidney (HEK) 293 cells, engineered to express alpha 7 nicotinic receptors or functional analogues thereof, e.g. human alpha 7 nicotinic receptors as described in Published International Application WO 94/20617.

Particularly preferred for this purpose are oocytes, e.g. *Xenopus* oocytes, engineered to express at the cell surface alpha 7 nicotinic receptors or functional analogues thereof. Preparation and maintenance *in vitro* of such oocytes expressing rat alpha 7 receptor subunits may be carried out as described in Séguéla et al., J. Neurosci. (1993) 13, 596-604. Example 1 below describes the production of *Xenopus* oocytes expressing at the cell surface human alpha 7 subunits as functional α-bungarotoxin-sensitive alpha 7 nicotinic receptors. Preparation of oocytes expressing such functional homomeric receptors is also described in WO 94/20617. Oocytes expressing alpha 7 nicotinic receptors of other species may be prepared in similar manner.

It will be appreciated that by employing, for example, mutant alpha 7 receptor subunit mRNAs for transfection, oocytes may also be prepared in similar manner expressing functional analogues of native alpha 7 receptors. Such functional analogues include receptors formed from chimeric subunits in which the extracellular domain portion of an alpha 7 subunit is joined to a portion of another protein capable of inserting into the cell membrane such that induction of Ca^{2+} influx in the presence of acetylcholine, or another agonist for the acetylcholine binding site of native alpha 7 nicotinic receptors, is maintained and can be modulated by Synaptica Peptide.

Functional analogues of alpha 7 nicotinic receptors suitable for use in a method of the invention may also be non-homomeric receptors which include 1 or more alpha 7 subunits and retain the following characteristics: (i) induction of Ca^{2+} flux in the presence of ACh, (ii) blockage of such Ca^{2+} flux by α-bungarotoxin at appropriate dose and (iii) modulation of such Ca^{2+} flux by Synaptica Peptide.

Induction of ion permeability through alpha 7 nicotinic receptors for the purpose of screening according to the invention may be achieved in any known manner for opening the calcium channel of such receptors. Preferably, acetylcholine or an alternative agonist capable of binding at the acetylcholine binding site will be employed. It may be particularly preferred to employ choline since alpha 7 nicotinic receptors have been found to have higher affinity for choline than α4β2 receptors and other nicotinic receptors. The anabasine analogue GTS-21 (2,4-dimethoxybenzylidene anabaseine) may alternatively be employed which has been reported to have high functional selectivity for homomeric alpha 7 nicotinic receptors compared to α4β2 nicotinic receptors.

As a preferred embodiment of the invention, there is provided a method of determining the ability of a compound to act as antagonist of the polypeptide of SEQ. ID. No. 1(Synaptica Peptide) which comprises:

(i) contacting said compound with an alpha 7 nicotinic receptor or a functional

analogue thereof in the presence of Synaptica Peptide or a functional analogue of said peptide under conditions whereby in the absence of said compound said peptide or functional analogue thereof modulates induced ion-flux through the receptor and

(ii) determining whether said compound antagonises the modulatory effect of said peptide or functional analogue thereof on the induced ion-flux,

wherein if said receptor is a native alpha 7 nicotinic receptor in its normal membrane environment, it is identified by means of inhibition by an alpha 7 nicotinic receptor blocker.

The ion flux which is determined in such a method may be Ca^{2+} ion flux. However, it will be appreciated that other ions capable of passing through the calcium channel of alpha 7 nicotinic receptors may be employed, e.g. ^{86}Rb ion flux may be determined as described in Published International Applications WO 91/15602 and WO 94/20617. The modulatory effect observed in the absence of the test compound may be enhancement and/or reduction of the ion flux depending on the dosage and time of application of Synaptica Peptide or the functional analogue thereof.

Thus, a particularly preferred embodiment of the invention is a method for determining an antagonist of Synaptica Peptide which comprises:

(i) providing cultured cells, preferably oocytes, engineered to express alpha 7 nicotinic receptors or functional analogues thereof at their outer surface in a medium containing Ca^2, said cells not expressing any other Ca^{2+}permeable receptor;

(ii) contacting said cells with (a) the compound to be tested, (b) means to induce Ca^{2+} flux through said receptors or analogues, preferably acetylcholine or an alternative agonist capable of binding at the acetylcholine binding site of said receptors or analogues in an amount sufficient to induce Ca^{2+} permeability and (c) Synaptica Peptide or a functional analogue thereof in an amount sufficient to modulate Ca^{2+} flux through said receptors; and

(iii) determining whether the test compound inhibits enhancement or reduction of Ca^{2+} flux observed in the presence of said peptide or analogue minus the test compound.

Synaptica Peptide or the functional analogue thereof will preferably be added to the culture medium so that there is no pre-incubation with the peptide or analogue prior to induction of Ca^{2+} flux and at a dose which initially produces enhancement of Ca^{2+}flux in the absence of the test compound. A suitable concentration of Synaptica Peptide or the chosen functional analogue thereof may be readily determined by initial experimentation. A concentration of Synaptica Peptide of about 0.001μM has been found suitable with *Xenopus* oocytes expressing human alpha 7 nicotinic receptors (see Example 1). Suitable concentrations of agonist for Ca^{2+} channel opening may also be readily determined. A concentration of acetylcholine of about 50 μM to 100 μM has been found suitable with *Xenopus* oocytes expressing human alpha 7 nicotinic receptors (see Example 1). Calcium influx can be followed by measuring change in membrane potential or by detection of intracellular Ca^{2+} ions using a Ca^{2+} detection agent such as fura and UV/visible or fluorescence spectroscopy. As indicated above, instead of Ca^{2+} ions, any other nicotinic receptor permeable ions may alternatively be employed including Na^+, K^+, Ba^{2+} and ^{86}Rb ions.

Such a test protocol can, of course, be modified to alternatively identify a functional analogue of Synaptica Peptide, e.g. a functional variant of Synaptica Peptide. In this case, cells expressing alpha 7 nicotinic receptors or functional analogues thereof will be incubated with the compound under test in the absence of Synaptica Peptide or a functional analogue thereof and means provided to induce ion permeability. The compound will be capable of competitively binding with Synaptica Peptide to the chosen target receptors. Enhancement or reduction of ion influx compared to that observed in the absence of the test compound, which can be blocked by an alpha 7 receptor blocker such as α-bungarotoxin or a specific antibody, is indicative of a compound capable of mimicking the action of Synaptica Peptide at its modulatory site

on the alpha 7 nicotinic receptor. Again, the cells employed will preferably be cells which express at their outer surface alpha 7 nicotinic receptors or functional analogues thereof in the absence of other Ca^{2+} permeable receptors, most preferably oocytes expressing alpha 7 nicotinic receptors or functional analogues thereof.

Identification of functional analogues and antagonists of Synaptica Peptide in accordance with the invention may also take the form of a neurite growth assay employing cultured neuronal cells upon which Synaptica Peptide can produce a toxic effect dependent on dosage and exposure time mediated via action on alpha 7 nicotinic receptors. Such cells are exemplified by GABA positive neurites in an organotypic tissue culture of neonate hippocampus, e.g. rat neonate hippocampus (see Example 2). Thus in a further embodiment, the present invention provides a method of identifying an antagonist of biological activity of Synaptica Peptide which comprises:

(i) providing cultured neurites upon which Synaptica Peptide is capable of producing a toxic effect dependent upon dose and exposure time;

(ii) incubating said neurites (a) in the presence of Synaptica Peptide or a functional analogue thereof at a dose sufficient to produce said toxic effect, (b) in the presence of the same dose of Synaptica Peptide or the chosen functional analogue thereof and the compound to be tested and (c) in the presence of the same dose of Synaptica Peptide or the chosen functional analogue thereof and an alpha 7 nicotinic receptor blocker such as α-bungarotoxin at a dose sufficient to block alpha 7 nicotinic receptors in said neurites; and

(iii) observing after a predetermined time whether said test compound inhibits a toxic effect of Synaptica Peptide on said neurites which is prevented by said blocker.

In step (iii), neurite ougrowth can be measured by an increase in the density of neurites and/or an increase in neurite length. An antibody may be employed to identify the neurites of interest. As indicated above, the cultured neurites may, for example, be GABA positive neurites present in an organotypic tissue culture of neonate

hippocampus. In this case, it will be found convenient, for example, to treat the tissue culture with the biotinylated and amidated analogue of Synaptica Peptide (see Table 1 in Example 2).

A modification of such a neurite growth assay may be employed to identify functional analogues of Synaptica Peptide. Thus, the present invention also provides a method of identifying a functional analogue of Synaptica Peptide, e.g. a functional variant thereof, which comprises:

(i) providing cultured neurites upon which Synaptica Peptide is capable of producing a toxic effect dependent upon dose and exposure time;

(ii) treating said neurites with (a) the compound to be tested and (b) the compound to be tested and an alpha 7 nicotinic receptor blocker at a dose sufficient to block alpha 7 nicotinic receptors in said neurites; and

(iii) determining whether the test compound produces a toxic effect on said neurites which is prevented by said blocker, said compound being capable of competitively binding with Synaptica Peptide to said receptors.

In a further aspect of the invention, there is provided a method for determining the ability of a compound to act as an antagonist of Synaptica Peptide, which comprises determining whether said compound can inhibit the action of Synaptica Peptide or a functional analogue thereof on alpha 7 nicotinic receptors bound to a support or presented at the surface of cells, e.g. human SH-SY5Y neuroblastoma cells, or cell membranes. Such a method may, for example, take the form of a binding assay and/or a cell viability assay (see Example 3). Compounds may, for example, be screened for (i) ability to inhibit decrease in binding of α-bungarotoxin or a functionally equivalent protein to the cells in the presence of Synaptica Peptide or a functional analogue thereof and/or (ii) ability to inhibit decrease of cell viability in the presence of Synaptica Peptide or a functional analogue thereof. Compounds may alternatively be screened for ability to mimic such effects of Synaptica Peptide on alpha 7 nicotinic receptors. Again, it will be appreciated that such compounds which represent functional analogues of

Synaptica Peptide will be capable of competitively binding with Synaptica Peptide to alpha 7 nicotinic receptors, for example such receptors presented by cells.

When screening for functional analogues or antagonists of Synaptica Peptide, it may be deemed desirable, or necessary, to initially screen for compounds capable of competitively binding with Synaptica Peptide to alpha 7 nicotinic receptors by a competitive binding assay. It will suffice for this purpose to employ alpha 7 nicotinic receptors or derivatives thereof, including individual alpha 7 receptor subunits or modifications thereof and portions of such subunits, provided binding ability for Synaptica Peptide is retained. Alpha 7 nicotinic receptors or derivatives thereof may be employed in membrane-bound form, e.g. as part of whole cells or an isolated membrane preparation, or in non-membrane bound form. Alpha 7 nicotinic receptors may be solubilised from membranes and isolated by affinity purification using α-bungarotoxin-conjugated Sepharose 4B as described by Drisdel and Green (*ibid*).

In a still further aspect, the present invention provides a method for screening a compound for ability to interact with an alpha 7 nicotinic receptor, which comprises contacting said compound with said receptor or a derivative thereof which retains a modulatory binding site for Synaptica Peptide in the presence of Synaptica Peptide or a functional peptide variant thereof and determining whether binding of Synaptica Peptide or the variant thereof is inhibited or prevented.

Preferably, the alpha 7 nicotinic receptor or derivative thereof will be non-membrane bound or presented by whole cells or a membrane preparation modified to present the target protein. For use in such a competitive binding assay, Synaptica Peptide or a variant thereof may be labelled with a revealing label for direct detection. Such a label may be any label conventionally employed for labelling proteins for detection, including a radioactive label, a fluorescence label, an enzyme label or detectable non-enzyme label such as biotin or an ESR or NMR detectable label.

Such an assay may take the form of an alpha 7 nicotinic receptor pull-down assay in which Synaptica Peptide or a derivative thereof is labelled with a ligand such as biotin and beads such as magnetic beads are provided linked to a receptor for said ligand. Such an assay may comprise the steps of:

(i) providing a preparation of alpha 7 nicotinic receptors, or derivatives thereof which retain a modulatory binding site for Synaptica Peptide, in non-membrane bound form or in the form of a cell lysate, e.g a neonate rat hippocampal cell lysate;

(ii) contacting said preparation with (a) Synaptica Peptide or a derivative thereof labelled with a ligand such that it retains ability to bind to the said modulatory site and (b) the compound to be tested;

(iii) additionally providing beads, e.g magnetic beads, linked to a receptor for said ligand label;

(iv) separating the beads from the preparation and determining whether there is reduction in the amount of captured alpha 7 nicotinic receptor or derivative thereof compared to the same assay carried out in the absence of the test compound.

Determination of any captured receptor or derivative thereof may take the form of Western blotting employing an antibody capable of identifying the receptor or derivative. Anti-alpha 7 antibodies for this purpose may be prepared by conventional techniques for obtaining antibodies to specific proteins and are obtainable from a number of sources. For example, a goat polyclonal anti-alpha 7 antibody is commercially available from Santa Cruz Biotechnology

In a still further aspect, the present invention provides a method of preparing a functional analogue or antagonist of Synaptica Peptide which comprises:

(i) identifying said functional analogue or antagonist by a method of the invention as described above and

(ii) synthesising said functional analogue or antagonist.

In the case of a compound so prepared, synthesis of the compound may be followed by incorporation into a pharmaceutical composition. The compound may be formulated together with a pharmaceutically acceptable carrier or diluent for passage across the blood-brain barrier. Means for such formulation may be conventional means well known in the pharmaceutical art.

The present invention also provides functional analogues and antagonists of Synaptica Peptide identified by a screening protocol of the invention and pharmaceutical compositions containing such analogues and antagonists together with a pharmaceutically acceptable carrier or diluent. As previously indicated, particularly preferred, for example, are antagonists thus identified which are capable of formulation for passage through the blood-brain barrier and thus inhibiting or preventing toxic non-enzymatic activity of AChE *in vivo*. Such compounds are envisaged as highly advantageous therapeutic agents for neurodegenerative disorders in view of their high selectivity for areas of the brain which may be affected by non-enzymatic function of AChE and ability to arrest or inhibit cell loss (see Figure 2 which summarises the proposed sequence of events leading to neuronal degeneration arising from activation of non-enzymatic activity of AChE).

In a still further aspect of the invention, there is thus provided use of an antagonist of Synaptica Peptide identified as described above for use in the preparation of a medicament for use in the treatment of a neurological disorder associated with non-enzymatic function of AChE, especially, for example, Alzheimer's Disease, Parkinson's Disease or Motor Neuron Disease.

The following examples illustrate the invention.

Examples

Example 1

Modulation by Synaptica Peptide of Ca^{2+} flux through alpha 7 nicotinic receptors at the surface of oocytes

Summary

With *Xenopus* oocytes transfected with alpha 7 subunit mRNA, a dose dependent modulatory effect can be observed on Ca^{2+} flux induced by acetylcholine. Synaptica Peptide alone has no effect on Ca^{2+} permeability. In the absence of pre-incubation with Synaptica Peptide, Ca^{2+} flux induced by acetylcholine may be observed to be initially enhanced followed by shutting off of Ca^{2+} channels. Such observations are indicative of Synaptica Peptide binding at a modulatory site on the alpha 7 nicotinic receptor since there is no other site on the external surface of the oocytes with which the peptide might be interacting.

Experimental procedures

Preparation of RNA transcripts and *Xenopus* oocyte injection

Complementary DNA encoding for the human alpha 7 nicotinic acetylcholine receptor was obtained as described by Peng et al. (Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in *Xenopus* oocytes (1994) Molecular Pharmacology, 45, 546-554). Total RNA was isolated from the human neuroblastoma cell line SH-SY5Y (purchased from the European Tissue Culture bank, UK) and a poly-(A)+RNA was isolated and used to construct a lambdaZap II cDNA library. Several million plaques were screened with a human alpha 7 cDNA probe. Hybridizations were performed at 42 °C in 40% formamide, 5x Denhardt's solution, 0.5% sodium dodecyl sulphate, 5x (0.18 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4, 1mM EDTA), 0.15 mg/ml denatured salmon sperm DNA. Membranes were washed and then exposed to Kodak XAR film. The selected clone was sequenced

and its sequence was shown to be identical to that registered by Peng et al. (EMBL accession number X70297).

Human alpha 7 receptor cDNA was ligated into a plasmid vector under the control of an SP6 promoter and *in vitro* transcribed alpha 7 subunit mRNA was used to transfect *Xenopus* oocytes. The RNA synthesis mixture was diluted (1:50) into RNA-free distilled water and then injected into stage V-VI *Xenopus* oocytes, which had been isolated and defolliculated manually. Injected oocytes were maintained for up to a week at 18°C in Barth's solution at pH 7.2 containing (mM): 88 NaCl, 1 KCl, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)$, 2.5 $NaHCO_3$, 0.5 theophylline, 10 HEPES plus 0.1 $\mu g/ml^{-1}$ gentamicin sulphate, 0.01 mg/ml^{-1} streptomycin sulphate and 0.01 mg/ml^{-1} penicillin-G.

Electrophysiology

Recordings were made 1 to 7 days following injection of RNA and at room temperature. Whole cell currents were measured by two microlectrode voltage clamp (Geneclamp 500B, Axon Instruments) using 0.6-2.5 MΩ agarose-cushioned electrodes containing 3 M KCl. Oocytes were placed in a 100 µl bath, which was gravity perfused continuously at 4 ml/min with Ringer's solution (mM: 115 NaCl, 2.5 KCl, 10 HEPES, 1.8 BaCl, pH 7.2).Agents to be applied to the oocytes were dissolved in the perfusion solution and applied by gravity perfusion using a manually-activated valve.

Functional alpha 7 nicotinic receptors were shown to be expressed after 24 hours post-injection of mRNA by monitoring ACh-induced currents. ACh currents were comparable with those mediated by native alpha 7 nicotinic receptors in their normal membrane environment (currents rectify inwardly, EC_{50} for ACh is around 100 µM with a Hill coefficient (nH) of 2; see Figures 3 and 4). 50 µM or 100 µM ACh was used for all tests with Synaptica Peptide.

Treatments with Synaptica Peptide

Application of Synaptica Peptide on its own to oocytes presenting alpha 7 nicotinic

receptors did not modify whole membrane currents. When Synaptica Peptide was applied to such oocytes at 0.001 μM with ACh, increased Ca^{2+} flux was indicated (see Figure 5).

Application of Synaptica Peptide followed after 2 minutes by co-application of Synaptica Peptide and ACh caused inhibition of ACh-mediated responses (see Figure 6). Further experiments were carried out with application of Synaptica Peptide at different concentrations in the range 10^{-12} to 5×10^{-6} with no pre-incubation or pre-incubation for 2 minutes (see Figure 7). When a high dose of Synaptica Peptide was employed, the enhancement of ACh-evoked response observed with low doses of the peptide was reversed. This trend occurred at lower concentrations of Synaptica Peptide if the peptide was given 2 minutes before application of ACh thereby effectively increasing the local concentration. Synaptica Peptide effects were found to be reversible but recovery was slow. These results are consistent with Synaptica Peptide acting at a modulatory site on alpha 7 nicotinic receptors and are also consistent with previous studies of the effect of Synaptica Peptide on ACh-induced calcium entry into neurons of neonate rat hippocampus and substantia nigra cultured *in vitro*.

Figure 8 shows additional data obtained by intracellular recordings in *Xenopus* oocytes expressing human alpha 7 nicotinic receptors. In these studies, intracellular recordings with application of 100 μM ACh showed as expected opening of Ca^{2+}channels. When 10 μM of Synaptica Peptide was applied without ACh, no Ca^{2+} current was observed. Addition of 10 μM Synaptica Peptide followed by after 30 seconds 100 μM ACh gave a reduced current compared to that observed with ACh alone. This is consistent with so much calcium entering the neurons, the calcium channels are shut off leading to reduction in the observed current.

Identification of agonists and antagonists for the modulatory action of Synaptica Peptide on alpha 7 nicotinic receptors

The protocols described above with *Xenopus* oocytes may be modified to provide an

advantageous system for identify functional analogues and antagonists of Synaptica Peptide. For example, a test compound may be substituted for Synaptica Peptide to determine if it will mimic the action of Synaptica Peptide on the alpha 7 nicotinic receptors. Alternatively, the test compound may be added with Synaptica Peptide to determine whether it will inhibit the effect of Synaptica Peptide in enhancing or inhibiting ACh-induced Ca^{2+} flux.

Example 2

Action of BamBi Peptide on GABA-containing neurons of neonatal hippocampus

Summary

In organotypic tissue culture of neonatal rat hippocampus, the biotinylated and amidated version of Synaptica Peptide (BamBi Peptide) has been found to have a significant toxic action on GABA-containing neurons.

Experimental procedures

(i) Isolation of hippocampal slices

Hippocampal slices were prepared from 7 day old Wistar rats. This age was chosen as the peak of neuronal migration has passed and the cytoarchitecture of the brain is already established, the brain is large enough for ready dissection and also brain nerve cells can survive explantation. Rats were decapitated by a scissor cut at the level of the foramen magnum. The skull was then cut along the midline from the base to the front and two horizontal cuts were made at the level of the ears. The skull was peeled away, cranial nerves cut and the brain removed and placed on the dorsal surface on a sterile petri dish on ice. A longitudinal cut was made through the medial cortex following the borders of the hippocampus caudally and the cortex was folded aside. A single cut was made through the cingulate cortex and underlying fimbria to prise the hippocampus free of the thalmus. Excess cortex and ahering meninges were removed before the hippocampus was placed ventral surface down on a McIlwain tissue chopper.

Coronal sections (400µm) were cut and individual slices were separated with the aid of a dissecting microscope and fine spatulas. These slices were placed at 4°C in fresh filtered Dulbecco's minimal essential medium (DMEM) for 1.5 hours. Leaving the slices in a balanced salt solution at 4°C allowed tissue debris and potentially toxic substances such as excitory amino acids to diffuse away and ruptured cell membranes to reseal.

(ii) Mounting of the tissue sections

400 µM hippocampal slices were attached to cleaned sterile poly-d-lysine coated coverslips by means of a plasma clot, formed by mixing a solution of chicken plasma (lyophilised chicken plasma 20µl, reconstituted in 5 ml distilled water) with bovine thrombin (0.8 mg/ml). The tissue section was placed on a 25 µl drop of plasma on a coverslip and a 20 µl drop of thrombin was then placed adjacent to the plasma drop. The two solutions were gently mixed in a circular manner until the clot covered the entire coverslip with the section held in the centre. After mounting the sections, the coverslips were refrigerated for 1.5 hours to allow the clot to set.

(iii) Maintaining organotypic slice cultures

Once the plasma clots had set thus holding the hippocampal slices in place, each coverslip was placed in a diagonal sided tube with a vented lid to assure adequate aeration avoid necrosis. Serum containing media (200µl) was added to each tube and the tubes placed horizontally in a humidified incubator (37 °C, 5 % CO_2/95% O_2) for 48 hours.

Following this period, the medium was replaced with 500µl of fresh filtered serum containing media and cultures were placed on a roller drum and replaced in the incubator at a 5° angle rotating at 10 revolutions per hour. This positioning and rotation of the cultures was important for two reasons. First, the cultures spent half the incubation time exposed to culture media and half to air, thus ensuring adequate aeration, Secondly, the rotation was important for thinning the cultures.

Cytostatic solutions were added to the media for the entire cultivation period to prevent over-proliferation of non-neuronal cells. The anti-mitotic substances use were uridine and cytosine-β-D-furanoside, both at 1 mM. Culture media was changed once a week by tipping out the old media and replacing it with 500µl of fresh filtered media warmed to 37°C. Cultivation was continued for 14 days prior to treatment.

(iv) Synthesis of BamBi Peptide

BamBi peptide (1µM) was prepared by solid phase synthesis employing amide resin from Novabiochem. Biotin was added using HBTU as a coupling reagent and "easylink" NHSbiotin from Pierce & Warriner.

(v) Treatments

BamBi Peptide (1 µM) either in the absence of α-bungarotoxin or in the presence of α-bungarotoxin (1 µM) was added in serum-free media following a period of 24 hours serum starvation. Treatments were for 1 hour to 14 days at 37°C on the roller drum.

(vi) Immunocytochemical identification of a microtubule-associated protein (MAP-2) and GABA positive neurons

The microtubule-associated proteins (MAPs) of brain exhibit various properties that suggest that they are important in the growth and stabilization of axons and dendrites during neuronal morphogenesis. MAP-2 is highly concentrated in neurons as one of the major components of microtubules and so is used as a stringent marker for neurons in the central nervous system.

By immunocytochemistry, it has been shown that in adult rat two high molecular weight forms of MAP-2 (MAP-2a and MAP-2b) are abundant in neuronal perikaraya and dendrite. A third splice variant (MAP-2c) is absent in the adult but represents a juvenile form that is abundant during embryonic life. MAP-2a, MAP-2b and MAP-2c are all present at birth, although during subsequent weeks MAP-2b increases transiently reflecting increased dendritic growth, After this critical stage of development, MAP-2c

finally disappears towards adulthood.

A monoclonal anti-MAP-2 (Roche) which specifically identifies MAP-2a and MAP-2b was used to measure dendrite length in treated tissue cultures. An anti-glutamic acid decarboxylase antibody (GAD monoclonal antibody; Chemicon) was use to identify GABA positive neurons.

On completion of the incubation period (14 day cultivation period and treatment period), immunocytochemical staining for MAP-2 and/or GAD was performed according to the biotin-avidin-peroxidase method. Cultures were cultivated for 14 days before being fixed in 4% formaldehyde for 1 hour at room temperature. In order to block any non-specific binding sites, cultures were incubated in 20 % goat serum in PBS for 1 hour at room temperature. Monoclonal antibodies were added to the cultures (100µl/culture in PBS plus 0.1% Triton X-100) lying in a humid chamber for 24 hours at 4°C and then incubated with biotinylated secondary (100µl/culture) for 1 hour at room temperature. Hydrogen peroxide was used to eliminate any endogenous peroxidase activity (0.3% hydrogen peroxide, 10% methanol in PBS) before finally cultures were incubated in avidin-biotin complex (ABC elite kit) and antibody detected using a diaminobenzidine (DAB) chromogen (0.05% DAB,0.015% hydrogen peroxide in PBS).

Light microscopy was used to calculate neurite outgrowth measurements and maximal neurite outgrowth was measured for 10 neurites from each culture. Recordings were averaged for each culture and within each culture group and expressed as a percentage of the media only group. Results from treatment groups were taken from at least 3 different culture days (i.e. made from different litters on different days). N numbers for stastical analysis were 11 for each group. Statistical analysis was by means of a one way Anova and a post-hoc Dunnetts mutiple comparison test.

Results

Table 1 below shows the effect of Bambi Peptide on GABA neurons in rat neonate

hippocampus at various concentrations and treatment times.

Table 1

BamBi Peptide conc.(M)	Time of treatment					
	1 hr	24 hrs	48 hrs	72 hrs	7 days	14 days
10^{-12}	ns	-	-	-	-	-
10^{-9}	pre-a*	pre-a	ns	ns	apo#	apo
10^{-8}	pre-a	pre-a	ns	ns	apo	apo
10^{-7}	-	apo	apo	apo	apo	apo
10^{-6}	pre-a	apo	apo	apo	apo	apo
10^{-5}	necrotic	necrotic	necrotic	necrotic	necrotic	necrotic

* pre-a=pre-apoptotic

apo = apototic

Use of hippocampal GABA neurons for identifying agonists and antagonists of Synaptica Peptide

The protocol discussed above can be adapted to identify functional analogues and antagonists of Synaptica Peptide. For this purpose, organotypic tissue cultures of rat neonatal hippocampus will be prepared in the same manner as above. For determination of a functional analogue of Synaptica Peptide, cultures will be treated with the test compound or the test compound together with α-bungarotoxin at a dose sufficient to block alpha 7 nicotinic receptors. Observation of toxic action on GABA neurons in the presence of the test compound, which is prevented by α-bungarotoxin, will be indicative of a functional analogue acting at the modulatory binding site for Synaptica Peptide on the alpha 7 nicotinic receptor.

For determination of a Synaptica Peptide antagonist, cultures will be treated with (a) BamBi Peptide alone at a dose sufficient to give a toxic action on GABA neurons (b) the same dose of BamBi peptide and α-bungarotoxin at a dose sufficient to block

alpha 7 nicotinic receptors and (c) the same dose of BamBi Peptide supplemented with the test compound.

Synaptica peptide may be similarly employed in neurite outgrowth assays.

Example 3

Binding of Synaptica Peptide (biotinylated and amidated) to alpha 7 nicotinic receptors on human SH-SY5Y neuroblastoma cells

SH-SY5Y (European Tissue Culture, UK) clonal cells were grown to confluence and then treated with biotinylated and amidated Synaptica Peptide (BamBi) at a range of concentrations (10^{-12} - 10^{-8} M) for three days, after which time they were harvested with sterile Phosphate Buffered Saline (PBS) and centrifuged at 240 g for 2 minutes. Pellets were then resuspended in 1 ml binding buffer (BB) consisting of 140 mM NaCl, 1 mM EDTA and 50 Tris-HCl at pH 7.4. Cells were counted using a haemocytometer and diluted in BB to give a final concentration of 2,000 000 cells per assay tube.

Binding Assay Protocol

All experiments were carried out with 100 µl of whole cell suspension and 50 µl of ^{125}I-α-bungarotoxin in a total volume of 250 µl. Non-specific binding was determined using 1 mM nicotine. For equilibrium competition binding assays, 2 nM ^{125}I-α-bungarotoxin was incubated with BamBi peptide at concentrations ranging from 1 pM to 3 mM. The same BB and cell number were used in saturation binding studies, but the concentration of ^{125}I-α-bungarotoxin varied between 0.1 to 5 nM. Cells were pre-incubated with nicotine for 30 minutes prior to the addition of ^{125}I-α-bungarotoxin. All reactions were performed in triplicate in borosilicate glass binding tubes and were incubated at room temperture for 90 minutes. Cells were washed twice with 4 ml ice cold binding saline to stop the reaction. Bound and free fractions were separated by vacuum filtration (Millipore) through glass microfibre filters

(GF/C, Whatman). Radioactivity was counted using a gamma counter.

Cell Viability

To determine the effect of BamBi peptide on cell viability,500, 000 cells were seeded onto 12-well culture plates. When the cells reached confluency, 10^{-12} M to 10^{-8} M BamBi peptide was added to the cultures. The cultures were grown in the presence of the peptide for up to 3 days. Cell death induced by BamBi peptide was assessed by tryptan blue dye exclusion. The cell cultures were stained immediately with 1.5 % tryptan blue for 10 min at room temperature and then rinsed with physiological saline. Cells stained with tryptan blue were considered nonviable. At least 200 cells were counted to determine viability for each culture well. In each experiment, cell counts on five wells were averaged to obtain the mean ± sem.

Results

BamBi peptide was found to produce a dose-dependent decrease in ^{125}I-α-bungarotoxin binding to SH-SY5Y cells as shown in Figure 9. The effect was reversed in the presence of 1 mM nicotine.

BamBi Peptide produced an acute and significant decrease in cell viability after 24 hours at all concentrations tested. However, this effect was reversible and after 72 - 90 hours cell numbers were similar to those of control cultures (see Figure 10).

Use of SH-SY5Y cells for identifying agonists and antagonists of Synaptica peptide.

Compounds may be screened for ability to mimic the above-noted effects of Bambi Peptide on SH-SY5Y cells or the ability to inhibit the same effects of Synaptica Peptide or a known functional analogue thereof.

Example 4

Pull-down of Alpha 7 nicotinic receptors using biotin-labelled Synaptica Peptide

Summary

Biotin-labelled Synaptica Peptide combined with use of magnetic beads carrying

steptavidin can be used to pull-down receptors from a rat neonate hippocampal lysate which are recognised by an anti-alpha 7 antibody. This provides further evidence that the target site for the non-enzymic function of AChE as mediated by Synaptica Peptide is the alpha 7 nicotinic receptor.

Experimental procedure

Biotinylated Synaptica Peptide was incubated with streptavidin-coated magnetic beads. The beads were washed to remove unbound peptide and incubated with a rat neonate hippocampal cell lysate. The beads were then washed to remove unbound material and protein eluted therefrom with low pH or SDS. The eluate was run on SDS-PAGE gels and Western blotting carried out using an anti-alpha 7 antibody. Pull down of alpha 7 nicotinic receptors was indicated. Such pull-down was not observed when adult rat hippocampus tissue was used.

CLAIMS:

1. Use of an alpha 7 nicotinic receptor or a functional analogue thereof to determine whether a compound is capable of acting as a functional analogue or antagonist of the polypeptide of SEQ. ID. No. 1 (Synaptica Peptide) on said receptor, wherein if said receptor is a native alpha 7 nicotinic receptor in its normal membrane environment, it is identified by means of inhibition by an alpha 7 nicotinic receptor blocker.

2. A method for determining the ability of a compound to act as an antagonist of the polypeptide of SEQ. ID. No. 1 (Synaptica Peptide), which comprises determining whether said compound can inhibit binding of Synaptica Peptide or a functional analogue thereof to its target site on an alpha 7 nicotinic receptor, or a functional analogue of said receptor, and thereby antagonise the modulatory effect of Synaptica Peptide or its analogue on induced ion flux through the receptor.

3. A method as claimed in claim 2 for determining the ability of a compound to act as an antagonist of Synaptica Peptide, which comprises :

(i) contacting the test compound with an alpha 7 nicotinic receptor or functional analogue thereof in the presence of Synaptica Peptide or a functional analogue of said peptide under conditions whereby in the absence of the test compound said peptide or functional analogue thereof modulates induced ion-flux through the receptor and

(ii) determining whether the test compound antagonises the modulatory effect of said peptide or functional analogue thereof on the induced ion flux,

wherein if said receptor is a native alpha 7 nicotinic receptor in its normal membrane environment, it is identified by means of inhibition by an alpha 7 nicotinic receptor blocker.

4. A method according to claim 3 wherein ion flux through the alpha 7

nicotinic receptor or functional analogue thereof is induced by acetylcholine or another agonist for opening of the ion channel of said receptor.

5. A method as claimed in claim 4 wherein ion flux through the alpha 7 nicotinic receptor or functional analogue thereof is induced by choline.

6. A use or method as claimed in any one of claims 1 to 4 wherein cultured cells expressing an alpha 7 nicotinic receptor or functional analogue thereof are employed.

7. A use or method according to claim 6 wherein said cells do not express any other Ca^{2+} permeable receptor.

8. A use or method according to claim 7 wherein said cells are oocytes.

9. A method for determining the ability of a compound to act as an antagonist of the polypeptide of SEQ. ID No. 1 (Synaptica Peptide), which comprises:

(i) providing cultured neurites upon which Synaptica Peptide is capable of producing a toxic effect dependent upon dose and exposure time;

(ii) incubating said neurites (a) in the presence of Synaptica Peptide or a functional analogue thereof at a dose sufficient to produce said toxic effect, (b) in the presence of the same dose of Synaptica Peptide or the chosen functional analogue thereof and the compound to be tested and (c) in the presence of the same dose of Synaptica Peptide or the chosen functional analogue thereof and an alpha 7 nicotinic receptor blocker at a dose sufficient to block alpha 7 nicotinic receptors; and

(iii) observing after a predetermined time whether said test compound

inhibits a toxic effect of Synaptica Peptide on said neurites which is prevented by said blocker.

10. A method for determining whether a compound is a functional analogue of the polypeptide of SEQ. ID no. 1 (Synaptica Peptide), which comprises:

(i) providing cultured neurites upon which Synaptica Peptide is capable of producing a toxic effect dependent upon dose and exposure time;

(ii) incubating said neurites with (a) the compound to be tested and (b) the compound to be tested and an alpha 7 nicotinic receptor blocker at a dose sufficient to block alpha 7 nicotinic receptors in said neurites; and

(iii) determining whether the test compound produces a toxic effect on said neurites which is prevented by said blocker, said compound being capable of competitively binding with Synaptica Peptide to said receptors.

11. A method according to claim 9 or claim 10 wherein said neurites are GABA positive neurites present in an organotypic tissue culture of neonatal hippocampus.

12. A method for determining the ability of a compound to act as an antagonist of the polypeptide of SEQ. ID No. 1 (Synaptica Peptide), which comprises determining whether said compound can inhibit the action of Synaptica Peptide or a functional analogue thereof on alpha 7 nicotinic receptors either bound to a support or presented at the surface of cells or cell membranes.

13. A method according to claim 12 wherein human SH-SY5Y

neuroblastoma cells are employed or membranes derived therefrom.

14. A method for screening a compound for ability to interact with an alpha 7 nicotinic receptor, which comprises contacting said compound with said receptor, or a derivative thereof which retains a modulatory binding site for Synaptica Peptide, in the presence of Synaptica Peptide or a functional peptide variant thereof and determining whether binding of Synaptica Peptide or said variant thereof is inhibited or prevented.

15. A method as claimed in claim 14 wherein Synaptica Peptide or said variant thereof is labelled with a revealing label.

16. A method as claimed in claim 14 or claim 15 wherein said compound is found to bind to the modulatory binding site of Synaptica Peptide and its ability to act as a functional analogue or antagonist of Synaptica Peptide is determined.

17. A method of preparing a functional analogue or antagonist of Synaptica Peptide, which comprises:

(i) identifying said functional analogue or antagonist by a method according to any one of claims 1 to 13 and 16 and

(ii) synthesising said functional analogue or antagonist.

18. A method according to claim 17 wherein the compound synthesised is further incorporated into a pharmaceutical composition together with a pharmaceutically acceptable carrier or diluent.

19. A method according to claim 18 wherein said compound is an antagonist of Synaptica Peptide.

20. A functional analogue or antagonist of Synaptica Peptide identified by a method as claimed in any one of claims 1 to 13 and 16.

21. An antagonist of Synaptica Peptide as claimed in claim 20 which is capable of formulation for passage across the blood-brain barrier.

22. A pharmaceutical composition comprising a functional analogue or antagonist of Synaptica Peptide as claimed in claim 20 together with a pharmaceutically acceptable carrier or diluent.

23. A pharmaceutical composition as claimed in claim 22 comprising an antagonist as claimed in claim 21 together with a pharmaceutically acceptable carrier or diluent.

24. Use of an antagonist of Synaptica Peptide identified in accordance with any one of claims 1 to 13 and 16 for the preparation of a medicament for treatment of a neurological disorder associated with non-enzymatic action of acetylcholinesterase.

25. A use in accordance with claim 24 wherein said neurological disorder is Alzheimer's Disease.

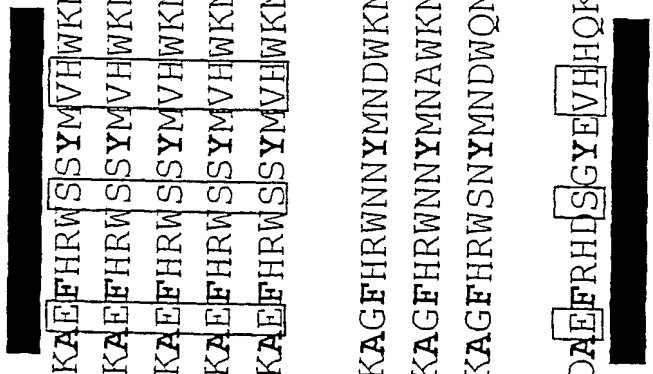
26. A use in accordance with claim 24 wherein said neurological disorder is Parkinson's Disease.

27. A use in accordance with claim 24 wherein said neurological disorder is Motor neuron Disease.

28. A method of treating a neurological disorder associated with non-enzymatic action of acetylcholinesterase which comprises administering an

antagonist of Synaptica Peptide identified in accordance with any one of claims 1 to 13 and 16.

Fig.1.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Rab AChE AFWNRFLPKLLSATDTLDEAERQWKAEFHRWSSYMVHWKNQF---DHY-SKQDRCSDL*
Mus AChE ----------LSATDTLDEAERQWKAEFHRWSSYMVHWKNQF---DHY-SKQERCSDL*
Rat AChE ----------LSATDTLDEAERQWKAEFHRWSSYMVHWKNQF---DHY-SKQERCSDL*
Bov AChE -FWNRFLPKLLNATDTLDEAERQWKAEFHRWSSYMVHWKNQF---DHY-SKQDRCSDL*
Hum BChE ----------TGNIDEAEWEWKAGFHRWNNYMNDWKNQF--NDYTSKKESCVGL*
Rab BChE ----------KVLEMTGNIDEAEQEWKAGFHRWNNYMNAWKNHF---NDYTSKKERCAGF*
Mus BChE ----------MTGDIDETEQEWKAGFHRWSNYMNDWQNQF---NDYTSKKESCTAL*
Hum Amyl ----------SGLTNIKTEEISEYKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL

Fig.2.

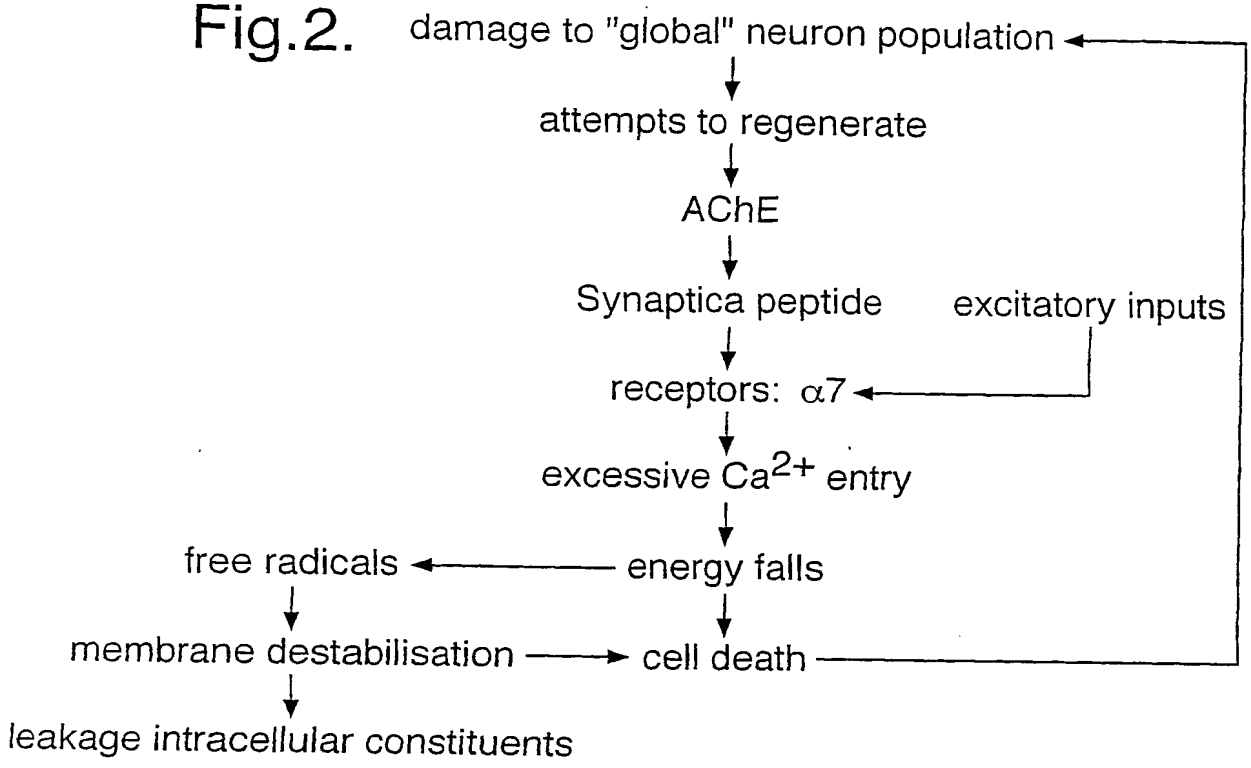

damage to "global" neuron population
attempts to regenerate
AChE
Synaptica peptide
excitatory inputs
receptors: α7
excessive Ca^{2+} entry
free radicals
energy falls
membrane destabilisation
cell death
leakage intracellular constituents

Fig.3.

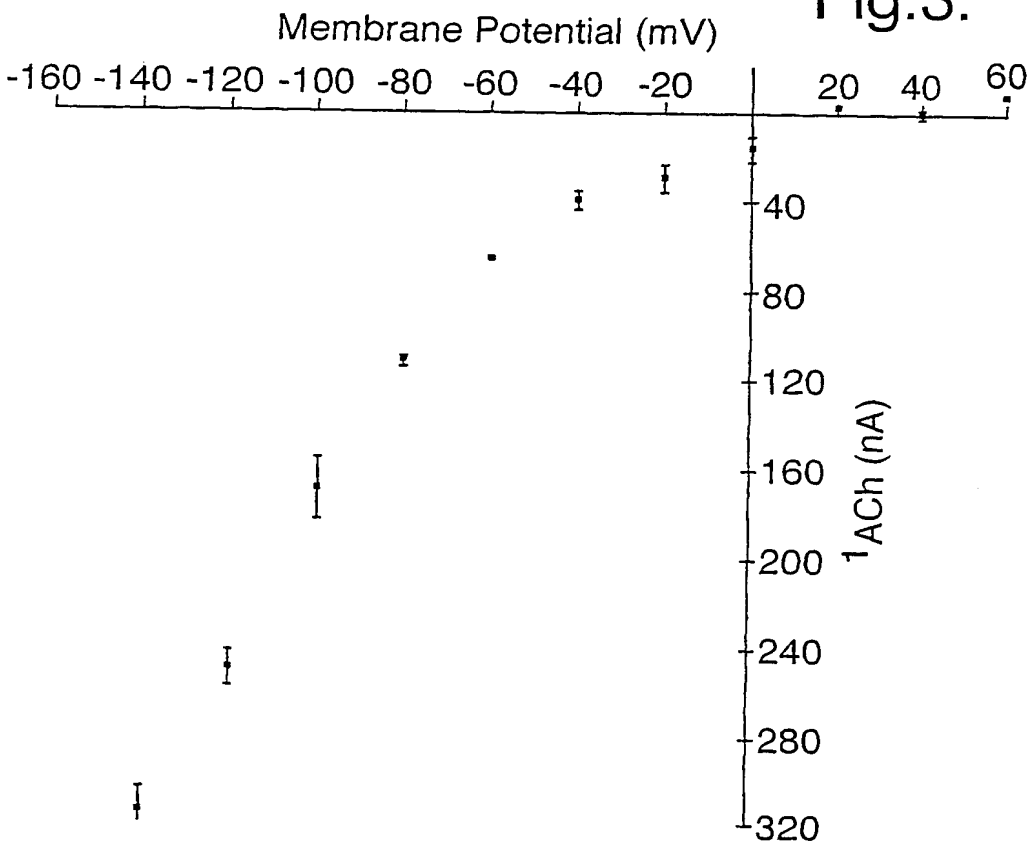

Membrane Potential (mV)
-160 -140 -120 -100 -80 -60 -40 -20 20 40 60
40
80
120
160
200
240
280
320
I_{ACh} (nA)

Fig.4.

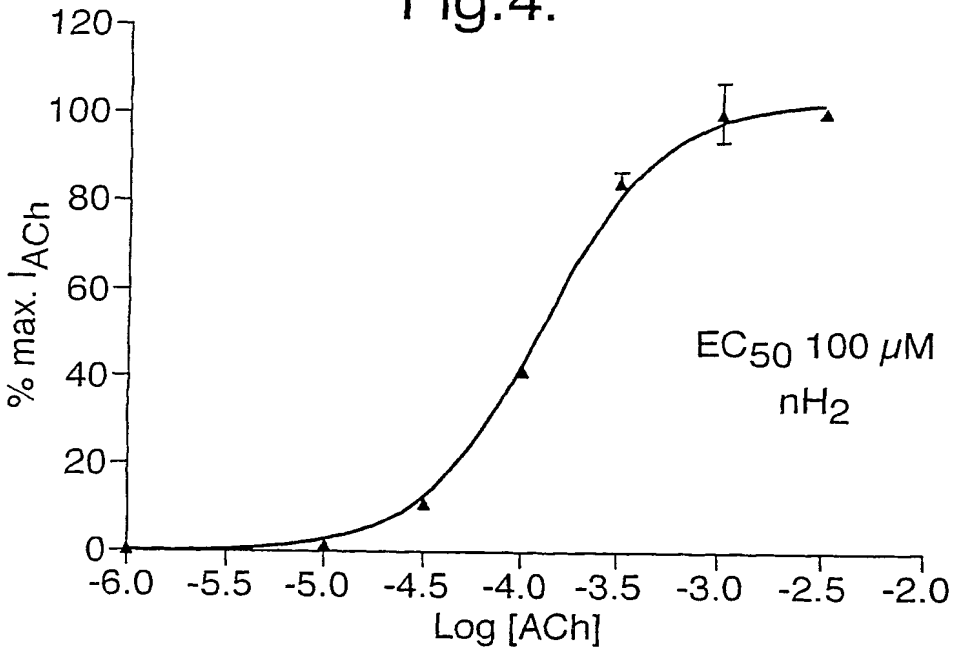

120
100
80
60
40
20
0
% max. I_{ACh}
EC_{50} 100 μM
nH_2
-6.0 -5.5 -5.0 -4.5 -4.0 -3.5 -3.0 -2.5 -2.0
Log [ACh]

Fig.5.

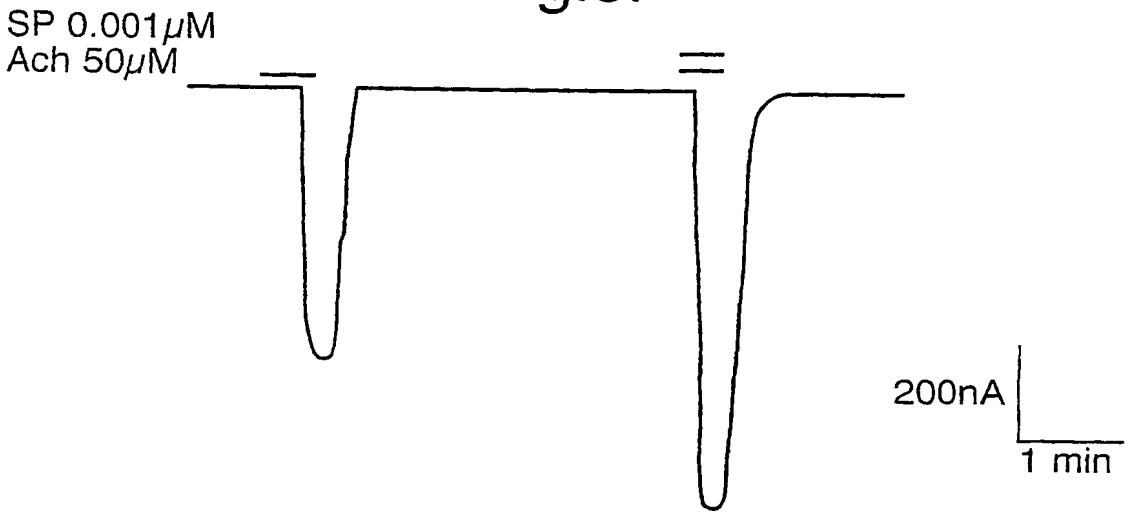

SP 0.001μM
Ach 50μM
200nA
1 min

Fig.6.

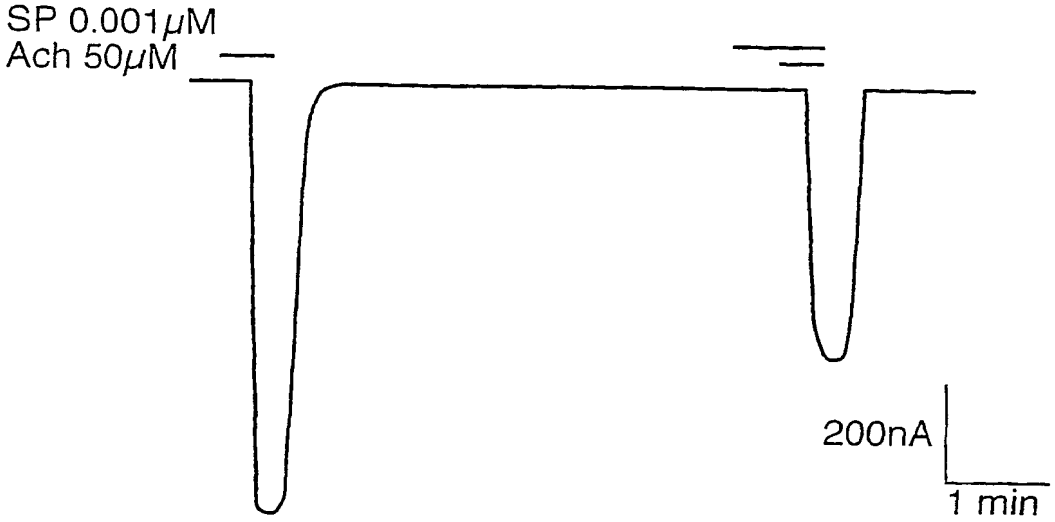

SP 0.001μM
Ach 50μM
200nA
1 min

Fig.7.

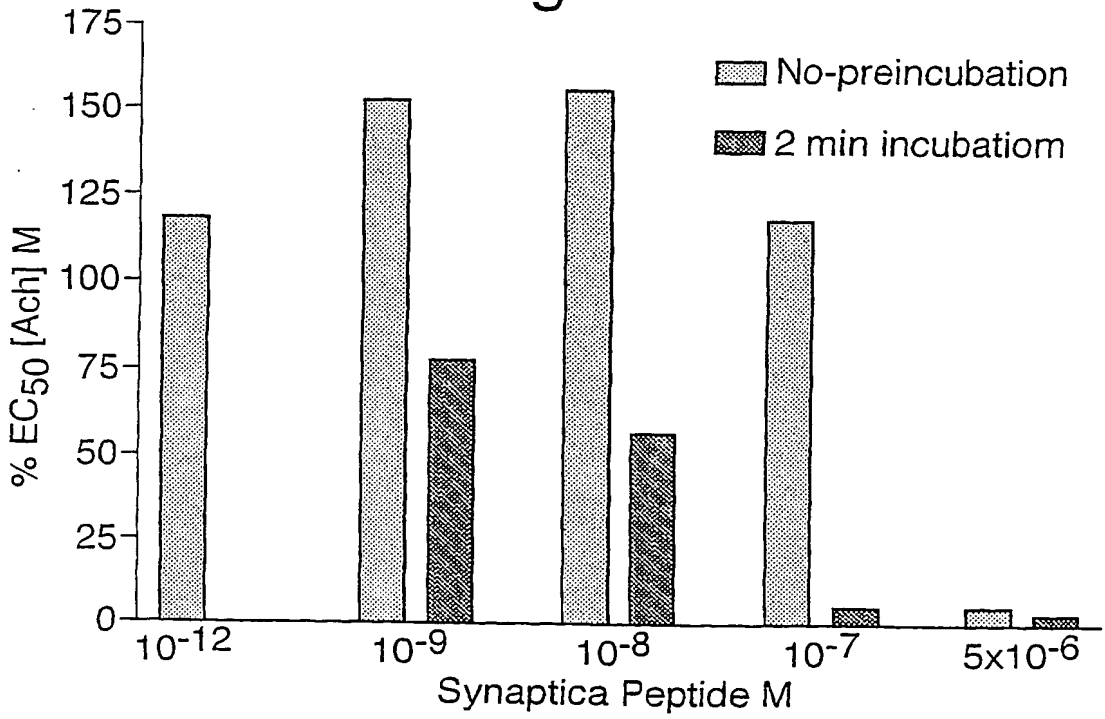

175
150
125
100
75
50
25
0
% EC50 [Ach] M
No-preincubation
2 min incubatiom
10-12
10-9
10-8
10-7
5x10-6
Synaptica Peptide M

Fig.8.

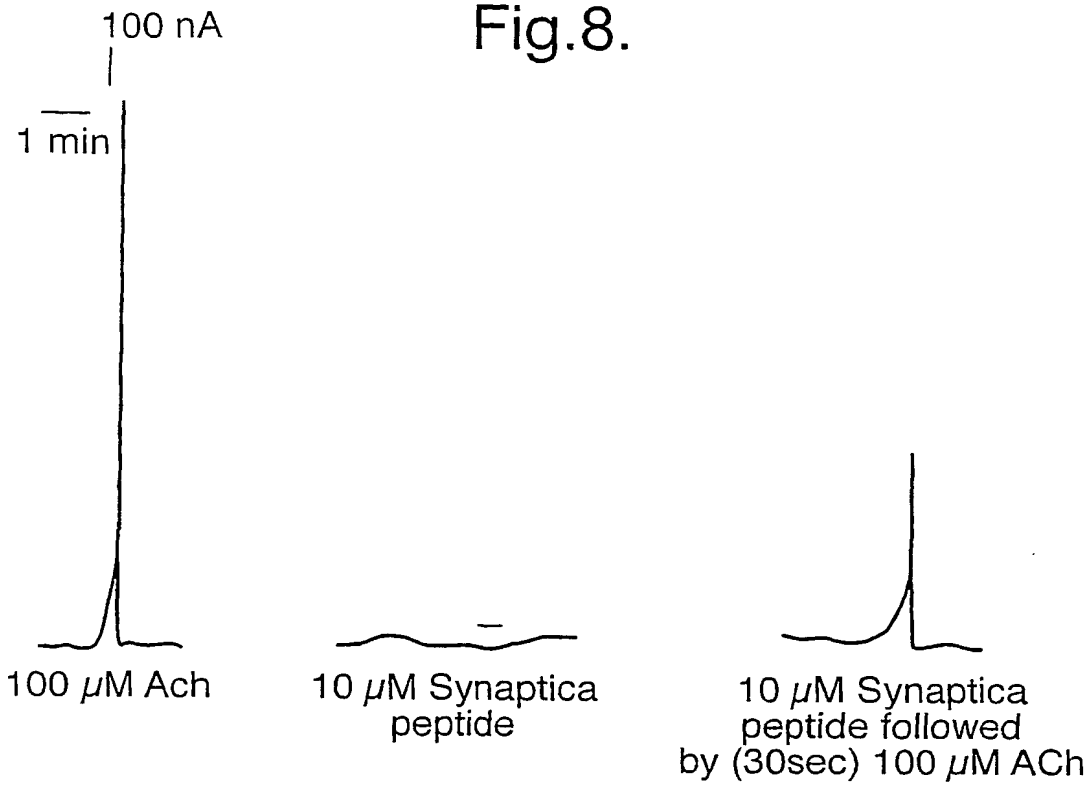

100 nA
1 min
100 μM Ach
10 μM Synaptica peptide
10 μM Synaptica peptide followed by (30sec) 100 μM ACh

Fig.9.

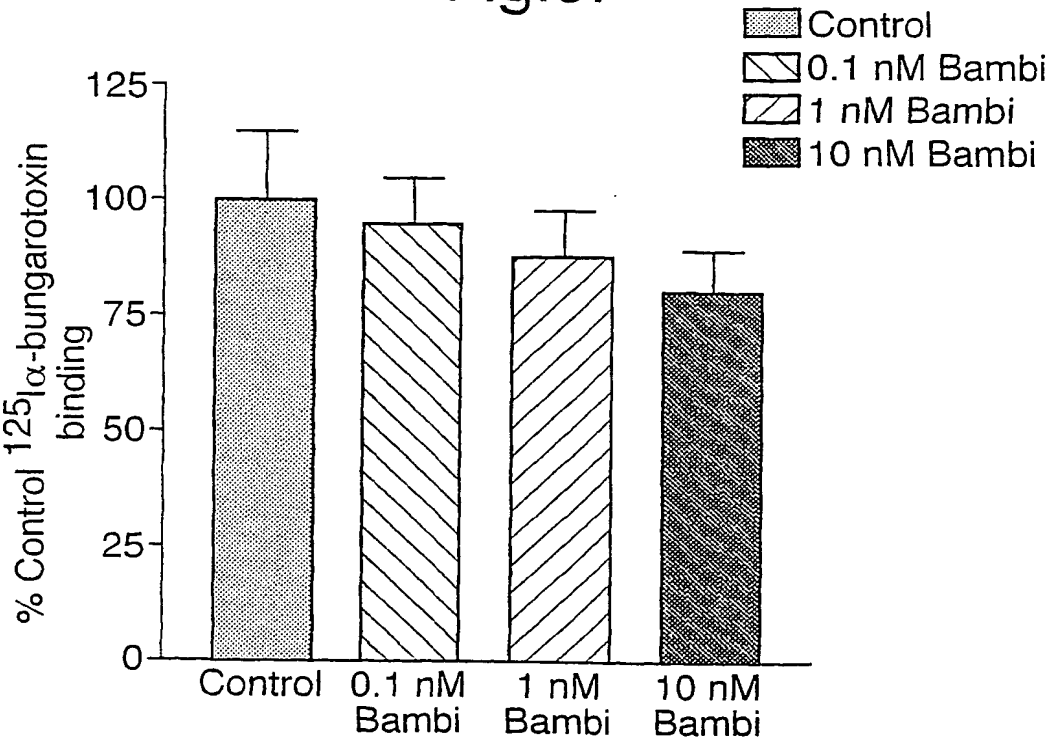
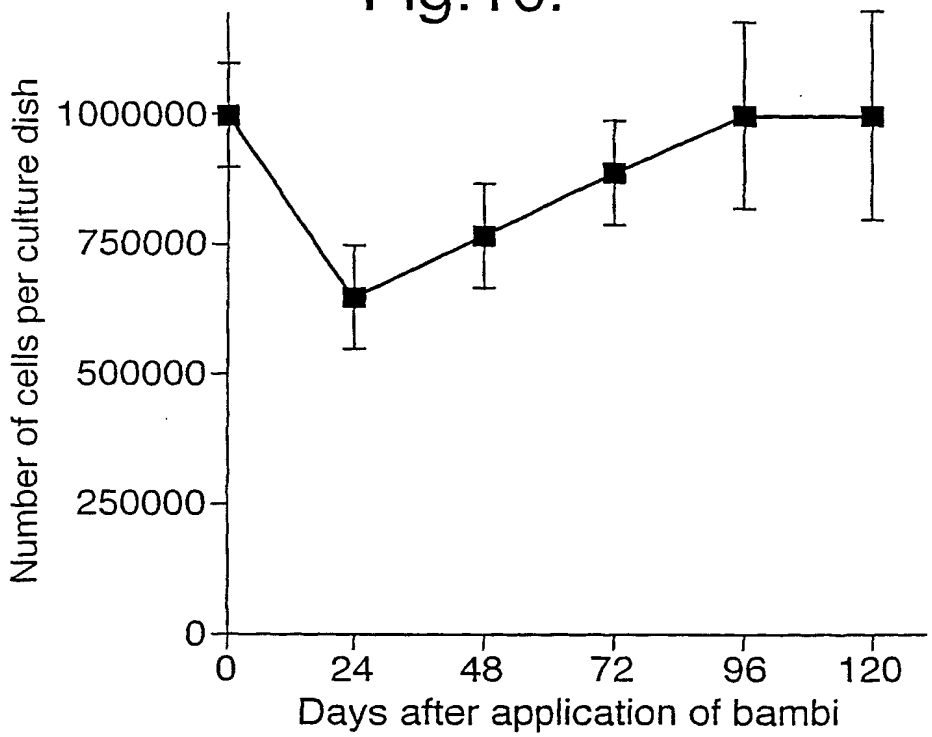

Control
0.1 nM Bambi
1 nM Bambi
10 nM Bambi
% Control 125I-α-bungarotoxin binding
125
100
75
50
25
0
Control
0.1 nM Bambi
1 nM Bambi
10 nM Bambi

Fig.10.


Number of cells per culture dish
1000000
750000
500000
250000
0
0
24
48
72
96
120
Days after application of bambi

INTERNATIONAL SEARCH REPORT

Inter ıal Application No
PCT/GB 01/01401

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/94 C12Q1/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 20617 A (ELLIOTT KATHRYN J ;HARPOLD MICHAEL M (US); SALK INST BIOTECH IND () 15 September 1994 (1994-09-15) cited in the application	1,6-8
A	the whole document	2-5
A	WO 97 35962 A (VAUX DAVID JOHN TALBUTT ;ISIS INNOVATION (GB); GREENFIELD SUSAN AD) 2 October 1997 (1997-10-02) cited in the application the whole document	1-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 August 2001	06/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gundlach, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/GB 01/01401

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALKONDON M. ET AL: "Choline is a selective agonist of alpha 7 nicotinic acetylcholine receptors in the rat brain neurons." EUROPEAN JOURNAL OF NEUROSCIENCE, (1997) 9/12 (2734-2742). , XP001023458	1,6
A	abstract page 2735, left-hand column, line 6 - line 13 page 2735, right-hand column, paragraph 2 -page 2736, left-hand column, paragraph 1 figure 2 page 2736, right-hand column, line 4 -page 2737, paragraph 1	10,11
A	WO 99 62505 A (CHEN ROBERT H ;REITZ ALLEN B (US); WANG HOAU YAN (US); DEMETER DAV) 9 December 1999 (1999-12-09) cited in the application abstract; claim 16	1-16

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 17-28

Present claims 17-28 relate to compounds or method using these compounds defined by reference to a desirable characteristic or property, namely being identifiable by the screening methods of claims 1-16.

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely the screening methods per se.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter al Application No
PCT/GB 01/01401

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9420617 A	15-09-1994	AU 694424 B	23-07-1998
		AU 6517394 A	26-09-1994
		CA 2155330 A	15-09-1994
		EP 0688361 A	27-12-1995
		GB 2286397 A,B	16-08-1995
		GB 2308121 A,B	18-06-1997
		JP 8507441 T	13-08-1996
		US 5837489 A	17-11-1998
		US 6022704 A	08-02-2000
		US 5910582 A	08-06-1999
WO 9735962 A	02-10-1997	EP 0896615 A	17-02-1999
		JP 2000509967 T	08-08-2000
WO 9962505 A	09-12-1999	AU 4543399 A	20-12-1999
		EP 1083889 A	21-03-2001

EXHIBIT 2

International Application WO 01/49107

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 July 2001 (12.07.2001)

PCT

(10) International Publication Number
WO 01/49107 A1

(51) International Patent Classification[7]: A01K 67/027, C07K 7/04, 14/47

(21) International Application Number: PCT/GB00/04991

(22) International Filing Date:
22 December 2000 (22.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9930825.6 30 December 1999 (30.12.1999) GB

(71) Applicant *(for all designated States except US)*: **SYNAPTICA LIMITED** [GB/GB]; Oxford Centre for Innovation, Mill Street, Oxford OX2 0JX (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GREENFIELD, Susan, Adele** [GB/GB]; University Department of Pharmacology, Mansfield Road, Oxford OX1 3QT (GB). **RAWLINS, John, Nicholas, Pepys** [GB/GB]; Department of Experimental Psychology, South Parks Road, Oxford OX1 3UD (GB). **DEACON, Robert, Michael, John** [GB/GB]; Department of Experimental Psychology, South Parks Road, Oxford OX1 3UD (GB).

(74) Agents: **IRVINE, Jonquil, Claire** et al.; J. A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/49107 A1

(54) Title: ANIMAL MODELS FOR NEURODEGENERATIVE DISEASE

(57) Abstract: The present invention provides animal models for neurodegenerative diseases, in particular, for example, Alzheimer's Disease, which rely on introduction into the brain of a biologically active fragment of acetylcholinesterase of sequence AEFHRWSSYMVHWK (SEQ. ID no.1), or an active variant thereof, to produce brain lesions.

ANIMAL MODELS FOR NEURODEGENERATIVE DISEASE

This invention relates to animal models for neurodegenerative disorders, in particular Alzheimer's disease, and to methods for providing them. In particular, the invention relates to the use of a peptide fragment from close to the C-terminus of acetylcholine esterase (AChE) for inducing cognition impairment so as to provide an animal model for neurodegenerative disorders.

Alzheimer's disease (AD) is a degenerative brain disease, the incidence of which rapidly increases with advancing age. Certain populations of brain cells progressively die, particularly but by no means exclusively those using acetylcholine as a neurotransmitter. Recently modern imaging techniques have revealed how the medial temporal lobe area, which contains the hippocampus (a vital structure for learning and memory generally in humans and for certain types of spatial learning in animals) progressively shrinks as Alzheimer's disease runs its course.

A sizeable minority of cases of Alzheimer's disease appear to have a genetic component (familial Alzheimer's) but the majority are sporadic occurrences with no known precipitating factors, although there are positive correlations with previous brain damage, low intelligence, and possibly aluminium concentration in drinking water. Cigarette smoking and folic acid appear to lower the incidence.

The principle symptoms of Alzheimer's disease are steadily progressive loss of cognitive faculties such as memory (particularly recent episodic memories), problems with language and speech such as difficulty in finding the right words, and attention. Multi-infarct dementia, the most common other form of dementia, often presents a similar clinical picture but as it is due to a series of small strokes its progression is more stepwise. Other clinically problematic associated symptoms of Alzheimer's are depression, aggression and eventually incontinence. Moderate to

advanced cases of Alzheimer's require 24 hour care, and thus the disease, which can affect 20% or more people over 80 years old, is enormously costly.

A number of proposals have previously been made for providing an animal model for Alzheimer's Disease. However, none of these have proved entirely satisfactory.

By way of example, transgenic mice have been produced which are genetically modified to produce larger than normal amounts of β-amyloid in the brain on the basis that this protein is found in plaques associated with development of AD. Some such mice show learning and memory deficits but have been criticised as acceptable models for AD since plaques can occur in normal ageing and the concentrations of β-amyloid do not necessarily correlate with the degree of dementia.

Other recent attempts to provide a good animal model for AD have been premised on the hypothesis that depletion of cholinergic neurons in relevant areas of the brain is of paramount importance. Post-mortem investigations of brains of AD patients have demonstrated that the cholinergic projection from the nucleus basalis of Meynert (NBM; also known as the nucleus basalis magnocellularis) to areas of the cerebral cortex is the pathway that is most early and severely affected in AD patients. Hence, a number of studies have looked at stereotaxic injection of neurotoxins into the NBM of rats to produce reduction in cortical cholinergic activity. A serious limitation of such studies accounting for unreliable behavioural results has previously been suggested to be lack of selectivity of the neurotoxin for cholinergic cells. To overcome this limitation, Bigl and Schliebs more recently proposed selective lesion of basal forebrain cholinergic neurons in rats by injection into the NBM of a cytotoxin (saporin) coupled to a monoclonal antibody to the nerve growth factor receptor associated with such neurons (Bigl,V. and Schliebs, R. (1998) Simulation of cortical deficits - a novel experimental approach to study pathogenic aspects of Alzheimer's disease, J. Neural. Transm. [Suppl] 54: 237-247). This approach to modelling AD is, however, also now considered open to

question as a reliable approach as the behavioural deficits observed can be inconsistent or insubstantial. Indeed, the above-noted paper of Bigl and Schlieb itself reports unsatisfactory behavioural results obtained by others using the same agent to cause cholinergic lesions in rat brains.

Significantly, although depletion of acetylcholine is prominent in AD, it is not the only characteristic. Another reason for disillusion with cholinergic models of AD has been poor clinical results from cholinergic therapy. Although tacrine, donepezil and rivastigmine, all AChE inhibitors, are the only currently licensed therapies for AD, this is primarily due to lack of more effective alternatives rather than their therapeutic efficacy, which is generally considered to be very limited. Also, toxicity and accompanying side effects can limit the usefulness of current therapies.

It has now been shown that cognitive impairment , e.g. attentional deficit, in rats reminiscent of that characteristic of AD patients can be produced by injection into the NBM and other brain areas of a 14 mer fragment of AChE having the sequence AEFHRWSSYMVHWK (SEQ. ID. no.1) or a biotinylated version of that peptide.Explanation for the effectiveness of this approach for mimicking AD can be founded on previous *in vitro* evidence implicating non-enzymatic action of AChE in the etiology of a number of neurological disorders including AD. However, the present specification for the first time presents evidence showing that a fragment of AChE alone can produce *in vivo* cellular degeneration and thereby neurological dysfunction reminiscent of neurological dysfunction associated with a known neurological disease. Importantly, this approach to modelling AD, in direct contradiction to the proposal of Bigl and Schliebs, cannot be attributed to a selective cholinergic deficit.

AChE is an enzyme whose classical or cholinergic role is to degrade extracellular acetylcholine. However, it has long been known that AChE can be found associated with non-cholinergic neurons. Consistent with this, in recent years there has been growing evidence that AChE has a non-enzymatic role, although the biochemical basis for this function remains to be fully elucidated.

Published International Application WO 97/35962 presents preliminary evidence indicating that the peptide of SEQ. ID no. 1 (referred to hereinafter for simplicity as AChE peptide) is capable of modulating induced Ca^{2+} flux into neurons, e.g. neurons of the substantia nigra in slices of guinea pig midbrain. It has been postulated that an *in vivo* counterpart of the peptide of SEQ. ID. no. 1 is responsible for mediating non-enzymatic function of AChE in the brain. It has been hypothesised that such non-enzymatic action of AchE underlies trophic function in developing brains but if activated in adult brains leads to neurodegenerative disorders. Based on knowledge of AChE neuronal location and *in vitro* studies with the peptide of SEQ. ID. No.1, this hypothesis of disease causation is currently of particular interest in relation to Alzheimer's Disease, Parkinson's Disease and Motor Neuron Disease (Greenfield, Spring research News (1997) 2-3; Greenfield, Brit. Med. J. (1998) 317, 19-26). However, as previously indicated above, direct evidence that the AChE peptide alone will produce neurodegenerative disease has been lacking.

WO 97/35692 does refer to infusion of a low dose of the AchE peptide via a cannula into one substantia nigra of a rat followed by systemic administration of amphetamine. Such treatment did give rise to behavioural disturbance, but such rats do not represent a useable model for neurodegeneration since they show increased neuronal activity rather than decrease of neuronal activity associated with cell death. More particularly, such animals do not provide any guidance for establishing a useful animal model for Alzheimer's Disease.

Significantly, AChE is present in the neuritic plaques and neurofibrillary tangles found in the cortex of Alzheimer brains (Carson et al. Brain. Res. (1991) 540, 204-208). However, interest in such plaques, as indicated above, has previously focussed on the β-amyloid component. Interestingly, as shown in Figure 1 of WO 97/35962, and in Figure 1 of the present specification, SEQ ID no. 1 (representing amino acid residues 535 to 548 of mature AChE) is conserved between AChE of different species, including human and rat AChE, and exhibits similarity to the N-terminal

region of β-amyloid peptide 1-42.

It has now been found that surprising histological changes occur when the AChE peptide is injected into the brains of rats. These are unlike anything normally seen with conventional neurochemical lesion. In particular, a lesion rapidly forms when the peptide is introduced into a region of the brain which is linked to the hippocampus by nerve projections, specifically the septal nuclei and/or the diagonal band of Broca (S/DB) region of the brain. This region provides major projections to the hippocampus (via the bundle of nerve fibres known as the fornix) and cingulate cortex. The lesion is an order of magnitude larger than that caused by a conventional neurotoxin such as NMDA, and the rapidity with which it forms is also remarkable (less than an hour; time course studies using MRI (magnetic resonance imaging) of the brain can illustrate this). Striking behavioural changes result which can be easily assessed by using standard tests of hippocampal function which are widely considered to be models of the functions affected in Alzheimer's disease. The physical changes, which include significant cell damage at and around the site of introduction of the peptide, can be observed by histological studies.

There is also provided herein for the first time evidence that attentional deficit reminiscent of that observed in Alzheimer's patients can be effectively modelled by injection of biotinylated AchE peptide (SEQ. Id no. 1) into the NBM of rats (see Example 4). Such deficit in cognitive function is associated with more subtle lesions than the AChE peptide lesions referred to above and cannot be correlated with loss of cortical cholinergic loss as made evident by comparison with comparable injections of NMDA. Importantly, comparable injection of NMDA was found to give a greater reduction in the level of cortical choline acetyltransferase (a measure of cholinergic loss) but without a selective effect on performance in the set attentional task (a serial choice reaction task as further discussed below).

Summary of the invention

The invention therefore provides in one aspect a method of providing an animal model for a neurodegenerative disease which comprises introducing, e.g. injecting, an effective amount of a peptide having the sequence:

AEFHRWSSYMVHWK (SEQ ID. no.1)

or an active variant of the peptide, into one or more sites in the brain of a non-human animal whereby said peptide causes cellular degeneration and thereby impairment of a testable brain function, wherein impairment of the same brain function in a human is indicative of a neurological disorder. It is predicted that such a method is applicable to modelling any neurological disorder associated with non-enzymatic function of AChE, in particular, for example, Alzheimer's Disease, Parkinson's Disease and Motor Neuron Disease. However, there is especial interest in adoption of this approach for modelling Alzheimer's Disease in which case the testable brain function of interest will be a cognitive function, e.g. attentional deficit. It will be appreciated that a method of the invention as described above above may further comprise testing for impairment of an appropriate brain function, e.g. by providing the animal with an attentional task to test for attentional impairment.

Prior to, simultaneously or after the peptide, a test agent may be administered. In this case, the animal model will be subsequently tested for the brain function of interest, e.g. attention, to determine whether the test agent inhibits, prevents or increases impairment of the relevant brain function. Of particular interest are compounds thus identified which will inhibit or prevent impairment of brain function associated with administration of the peptide alone and which can be formulated for passage across the blood-brain barrier.

The invention will be further described below with reference to the figures detailed below.

Brief description of the figures

In the attached figures:

Figure 1 shows a multiple sequence alignment of five AChE sequences, three BuChE sequences and the human amyloid precursor protein (Hum Amyl) at the region of interest. Hum AchE = human AchE; Rab AchE = rabbit AchE; Mus AchE = mouse AchE; Bov AchE = bovine AchE; Hum BuCHE = human butyrylcholinesterase (BuChE); Rab BuChE = rabbit BuChE; Mus BuChE = mouse BuChE. Residues in bold are conserved across all sequences. Boxed residues are shared by all AchEs and human amyloid precursor protein but by none of the BuChEs. The β-amyloid peptide 1-42 is shown by the boxed area. The bar above the alignment shows the position of the AchE peptide (SEQ. ID. No. 1). The bar below the alignment shows the position of the homologous peptide fragment at the N-terminus of β-amyloid 1-42.

Figure 2 is a graph showing the weight of rats (mean +/- SEM) on days 1 to 10 following injection with 2 μl 33 mM AchE peptide (also known as Peptide B), 2 μl 33 mM NMDA or 2μl water into the medial septum/vertical limb of the diagonal band of Broca (S/DB).

Figure 3 shows the results of T-maze tests pre-operatively and two weeks post-operatively for the same rats as described in Example 2. Veh= controls injected with water; NMDA = rats injected with NMDA; B= rats injected with AchE peptide.

Figure 4 shows the results of 20 massed T-maze trials as referred to in Example 2.

Figure 5 shows T-maze results for the same rats when a 45s delay was imposed between sample and choice trials as discussed in Example 2.

Figure 6 shows the locomotor activity for the rats referred to in Example 2 as tested on 2 successive days for 4 hours in standard locomotor activity cages following completion of the T-maze testing.

Figure 7 shows comparison of the weights of the rats referred to in Example 2 pre-operatively and I month post-operatively prior to

microscopical brain examination.

Figure 8 shows the arrangement of the septal nuclei and their connections with the hippocampus in diagrammatic form.

Figure 9 shows area of tissue loss in the medial septal region of rats anaesthetised and injected as described in Example 2 with AchE peptide (B), the equivalent peptide from BuChE (C), a scrambled version of AchE (ScrB), NMDA or water (W) (*p less than 0.05 vs water).

Figure 10 is a schematic representation of some ascending cholinergic pathways in the rat brain. Abbreviations: h, hippocampus; ms, medial septal nucleus; nb, nucleus basalis; nc, neocortex; nv1, nh1, nuclei of diagonal band, lie adjacent to medial septum.

Figure 11 shows a rat testing apparatus for performing a serial choice reaction task test for attention as described by Higgs et al., European J. Neuroscience (2000) 12, 1781-1788.

Figure 12 shows the result of decreasing light stimulus duration on ability of non-treated rats to perform an attentional task in apparatus as shown in Figure 11. The right panel shows the % of correct lever-light trials. The left panel shows the number of incorrect choices which were errors to the centre tray (which would have been correct had no light been presented).

Figure 13 is a line drawing of a coronal section of the right side of a rat brain to show the site of microinjection of AchE peptide as described in Example 4. The microinjection site within the NBM is shown as a dark spot at the base of the internal capsule and globus pallidus. Abbreviations: AT = anterior thalamus; C = cortex, CC=corpus callosum; CP = corpus striatum; F-fimbria-fornix; GP = globus pallidus; IC = internal capsule; Rt = reticular nucleus of the thalamus; VL= lateral ventricle; V3=third ventricle.

Figure 14 shows the results of testing rats in apparatus as shown in Figure 11 following injection into the NBM of 2 µl water, 2 µl 16.5 mM biotinylated AChE peptide (referred to in Figure 14 as Synaptica Peptide) or 2 µl 16.5 mM NMDA as described in Example 4. Results are

also shown for a fourth group of hippocampal lesioned rats. The left panel shows the proportion of correct trials as a proportion of the total trials, The right panel more specifically indicates where rats went on incorrect trials. Each column represents correct trials/correct trials plus incorrect trials to centre. The right panel thus shows the tendency for each group to go to the centre tray as if no light had been presented (rather than respond wrongly to the wrong side).

Detailed description

Of particular interest, for example, so far as an animal model for Alzheimer's disease is concerned is the introduction of the peptide into a site in the septohippocampal cholinergic system, preferably the S/DB region. A single injection here of a sufficient dose can have pronounced effects on behaviour. Alternatively or additionally, the peptide may, for example, be introduced into a site in the cortical cholinergic system, preferably in the nucleus basalis/substantia innominata.

As indicated above, an especially preferred embodiment comprises introducing AChE peptide or an active variant thereof, e.g. especially the biotinylated form of SEQ. ID. No. 1 or an active variant thereof, into the nucleus basalis magnocellularis region (or nucleus basalis of Meynert) of a non-human animal, preferably a rodent, so as to produce lesions which can be linked to attentional impairment. An appropriate concentration range for the peptide may be established by conventional histological methods for identifying lesions in animal brains and/or by recognised behavioural tests. A concentration of biotinylated AChE peptide as high as 16.5 mM has been employed satisfactorily in preliminary studies but more physiological doses may prove preferable,

The septohippocampal cholinergic system is a communication system which operates between the S/DB and the hippocampus, along connecting neurones (see Figure 8) The cortical cholinergic system is a separate cholinergic system. As previously indicated above, it starts in the nucleus basalis region of the brain. Neurons

within the NBM send long projections to most areas of the cerebral cortex, including the prefrontal and parietal regions, which are involved in attention. In rats, the nucleus basalis/substantia innominata regions overlap and are ill-defined. The term nucleus basalis magnocellulularis or nucleus basalis of Meynert (NBM) as used herein will therefore be understood to equate with the whole area emcompassing the NBM which provides projections to the cortex.

Both the cortical and septohippocampal cholinergic systems contribute to memory. Memory impairment in rats with lesions of the nucleus basalis/substantia innominata and medial septum including cell bodies of the cortical and septohippocampal cholinergic systems, respectively, have been compared previously (Miyamoto et al. Brain Research (1987) 419:,19 - 31). Memory impairment was observed in both cases, although this was far less marked in the case of lesions in the cortical cholinergic system. Nevertheless, as previously indicated above, the NBM is now viewed by the inventors in this instance as a preferred site at which the AChE peptide, or an active variant thereof, may be introduced to produce behavioural changes that represent behaviour in Alzheimer's disease.

A method of the invention for preparing an animal model of Alzheimer's Disease may include testing for one or more aspects of cognitive function known to be affected in Alzheimer's Disease. Peptide treated animals may thus be tested for one or more of impairment of memory, learning, attention and problem-solving. Particularly suitable for testing animals such as rats are working spatial memory tests, such as the T maze test described herein. Other standard tests which can be applied include for example the Morris water maze and the radial arm maze.

It is particularly preferred to combine production of peptide lesions in the NBM with testing for attentional deficit using apparatus providing a serial choice reaction task. Rats can be trained to perform simple attentional tasks, such as to push open a panel with their nose when a light flashes behind it and retrieve a food reward. Although sensitive to

treatments affecting attention, a failure to respond in such a test might also be due to an effect on performance. The treatment might for example cause sedation. A serial choice reaction task addresses this concern by providing for more than one stimulating event, for example lever-pressing by a rat may result in one of three events: a light flash from a left magazine or a right magazine or no light in which case the correct choice is a central magazine. Suitable apparatus for testing for attentional impairment in this manner has been described in Higgs et al., European J. Neuroscience (2000) 12, 1781-1788 (see also Figure 11).

Other behavioural functions which can be monitored include but are not limited to social behaviour, emotional reactivity, contextual conditioning, pre-pulse inhibition of startle reflex, two-way aversive conditioning and motivation as measured by food and water intake or sucrose preference.

As indicated above, the peptide employed in preparing an animal model in accordance with the invention may be the AchE peptide (SEQ. ID. No. 1) itself or an active variant thereof, including modified forms of that peptide having modified amino acid residues, e.g. the biotinylated form. Variants of the AchE peptide include peptides having one or two or a few amino acid substitutions and/or one or two or a few amino acid deletions and/or a one or two or a few additional amino acid residues. compared to SEQ. ID. No. 1. A suitable variant may for example have an N-terminal and/or C-terminal extension. It may be the *in vivo* counterpart of the peptide of SEQ. ID.no. 1.

Given SEQ. ID. No. 1 as a guide for comparison, it is a straightforward matter to make variant peptides and test them for efficacy in a method according to the invention. For example, one might start by testing a peptide which is identical to the AChE peptide except for one or two conservatively substituted amino acid residues. Conservative substitutions can be predicted on the basis of amino acid properties which are well characterised. Active variants of the AchE peptide for use in accordance with the invention may also possibly be determined by *in vitro*

tests of peptides for retention of calcium channel modulatory activity. For this purpose, guinea pig midbrain slices may, for example, be employed for electrophysiological studies as described previously in Published International Application no. WO. 97/35962. Alternatively, for example, organotypic tissue culture of hippocampal slices, e.g. from rats, may be used.

Suitable variants of the AChE peptide for use in the invention are expected to be peptides containing at least six amino acid residues and having at least 70% sequence identity with part or all of the AChE sequence above. Preferably, peptides for use in the invention are expected to contain at least 12 amino acid residues and have at least 90% sequence identity with SEQ. ID. No. 1.

As indicated above, subtle lesions in the NBM of rats giving rise to attentional deficit have been found to be achievable by use of biotinylated AchE peptide. However, it is envisaged that functionally equivalent lesions in the NBM may be achieved by use of other peptides as discussed above.

The source of the peptides described herein for use in the invention is not material to the invention. They may be for example synthetic peptides prepared by chemical synthesis, or they may be prepared from larger peptide or polypeptide molecules by enzymatic digestion, or they may be produced by recombinant techniques.

The chosen peptide will normally be administered by stereotaxic injection into an anaesthetised animal, although administration to conscious animals through implanted cannulae may sometimes be preferred, e.g. to examine acute effects (30 minutes duration) without anaesthesia. Alternatively, pressure microinjection or electrophoresis through a (glass) micropipette may be preferable for ionophoresis recordings.

In another aspect, the invention provides non-human mammals, e.g rodents, treated according to a method described herein. It will be appreciated from the discussion above that in a particularly

preferred embodiment such animals are known to exhibit impaired cognitive function, e.g. attentional deficit, as a result of a lesion in the brain, in particular the destruction of cells in or associated with the septohippocampal or cortical cholinergic system, especially for example destruction of cells in the NBM as a result of injection therein of biotinylated AchE peptide or an effective variant thereof. Although the examples below, relate to studies with rats, it will be appreciated that the invention is not confined to such animals but also extends to other animals which may be treated in accordance with the invention to produce neurological dysfunction, including, for example monkeys.

As indicated above, particularly favoured are animals according to the invention which represent models for Alzheimer's disease or at least an aspect of that disease. For instance, such models can be used to study potential cognition enhancing agents, or to test generally for agents having biological activity relating to neurodegeneration.

In addition to animal models for Alzheimer's disease, the invention also envisages, however, animal models for a range of neurodegenerative disorders, including but not limited to Parkinson's disease, motor neuron disease, and prion-related disorders such as bovine spongiform encephalopathy and Creutzfeldt-Jakob disease (CJD, including "new variant" CJD). For example, a rat injected with the AChE peptide described herein in the substantia nigra region of the brain may be useful as a model for Parkinson's disease and thus for testing reagents to assess their potential as therapeutic agents for treatment of Parkinson's disease.

As previously indicated above, a method of the invention may further comprise administering prior to, simultaneously, or after the peptide a test agent and determining whether said agent can inhibit, prevent or increase impairment of the testable brain function of interest, e.g. in the case of an animal model for Alzheimer's Disease, attention as determined preferably by, for example, performance of a serial choice reaction task, and/or can inhibit, prevent or increase cellular damage in the brain. The test agent may be a compound administered in any conventional manner

for a therapeutic agent whereby it can gain entry to the brain. Alternatively, it may be a cellular transplant introduced into the brain.

In a further aspect, there is provided a method of testing an agent for biological activity in a neurodegenerative disease, which method comprises administering the agent to an animal model as described herein and assessing the animal for any change (improvement or deterioration) associated with the brain lesion. Such assessment will comprise determining whether said agent will inhibit, prevent or increase impairment of an appropriate testable brain function, e.g. a cognitive function such as attention or memory, and/ or determining whether there is any improvement or deterioration in cellular damage at the relevant site(s) in the brain.

A test agent identified as above which inhibits or prevents impairment of a testable brain function constitutes a further aspect of the invention. A pharmaceutical composition comprising such a test agent together with a pharmaceutically acceptable carrier or diluent also forms part of the invention.

A method of assessing a test agent as described above may further comprise synthesising a selected compound found to inhibit or prevent impairment of a testable brain function. Such synthesis may be followed by incorporation of the synthesised compound into a pharmaceutical composition. It will be appreciated that of particular interest are such compounds which can be formulated to pass through the blood-brain barrier. Such compounds may be of therapeutic use in treating a neurological disorder such as Alzheimer's Disease.

In a further aspect, there is thus provided use of an agent selected in accordance with the invention as above which inhibits or prevents impairment of an appropriate testable brain function of the animal model for the manufacture of a medicament for use in the treatment of a neurological disorder. In a preferred embodiment, such use is use of an agent which has been shown to inhibit or prevent impairment of a cognitive function , e.g. attention, associated with injection of an appropriate peptide into the brain of a rat to model Alzheimer's Disease.

In more detail, the findings on which this invention is based are as follows.

Initial injection of the AChE peptide into the dorsal hippocampus of rats was found to cause cell loss in a limited area around the site of injection and cortical damage around the injection tract. The neuronal loss in the hippocampus itself, however, appeared less than from injections of the known "reference" neurotoxin N-methyl-D-aspartate (NMDA) in equivalent concentrations.

The comparatively small amount of histological damage after intrahippocampal AChE peptide injection suggested that in order to see any behavioural effects from AChE peptide given intracerebrally it would be necessary to inject it at multiple sites within the hippocampus. To produce a complete lesion of the hippocampus in a rat using the reference neurotoxin NMDA, 14 injections are required on each side of the hippocampus, making 28 in total. As noted above, the damage from AChE peptide was more localised than that from NMDA, so even more injections would probably be necessary. This would be technically difficult.

It was then discovered that by injecting the peptide into a different site, at the border of the medial septum and the diagonal band of Broca (S/DB) region of the brain, a much larger lesion could be produced which also affected the hippocampus via the fornix connecting system, even with only a single injection. Furthermore, the lesion could be identified using simple behavioural tests known to be affected by hippocampal dysfunction.

The medial septum is a small compact structure that has a powerful influence on other parts of the brain, and hence on behaviour. It projects nerve terminals (axons) into large regions of the hippocampus. The medial septum lies next to the vertical limb of the diagonal band of Broca (VDB), which innervates the cingulate cortex above the hippocampus and may play a role in some aspects of learning, memory,attention and emotional behaviour.

These results were unexpected as there was a major problem

anticipated with this approach. Selective chemical lesions of the septum normally produce much smaller effects than more traditional techniques involving mechanical or electrolytic destruction of brain tissue. These much smaller effects require very much more sophisticated tests to assess them, involving extensive animal training and complex apparatus, impractical for a workable animal model. This, however, proved not to be the case here.

Rats injected with AChE peptide in the S/DB showed a greater weight loss than controls after surgery, before recovering to near control weights. NMDA-treated rats showed a similar initial weight loss to AChE peptide-treated rats, but a much faster recovery over subsequent days (see Figure 2).

When tested for working spatial memory on a T-maze (on which all groups had been trained prior to operation, when they showed near-perfect performance) control rats continued their excellent performance, NMDA-treated rats showed an initial dip in performance but soon recovered to control levels, whereas AChE peptide-treated rats showed a larger drop than the NMDA-treated rats. Unlike NMDA-treated rats, the AChE peptide-treated group's performance did not recover significantly (see Figures 3, 4 and 5). Two rats (out of the six AChE peptide treated rats) were at virtually chance levels throughout testing, a characteristic sign of a large lesion in the hippocampal system.

When tested for locomotor activity, the AChE peptide treated rats were significantly hyperactive compared to controls (another sign of hippocampal dysfunction). The two rats which were worst on the T-maze were the most active in the locomotion test. NMDA-treated rats were slightly more active than controls but this was not statistically significant (see Figure 6).

At post-mortem, the two AChE peptide treated rats which were worst on the T maze and most hyperactive showed gross atrophy of the septum (see Figure 9) and of the fornix, the band of nerve fibres which carries projections from the medial septum to the hippocampus.

These experiments indicated that the AChE peptide can

cause considerable central nervous system damage, which appears to be an order of magnitude greater than that of the benchmark neurotoxin NMDA. Cytotoxins normally only affect cell bodies; the gross damage to the fibre tracts of the fornix suggests a different or additional mode of action of AChE peptide. Studies suggest that the S/DB area of the brain may be particularly susceptible to damage. These striking results were unexpected and could not have been predicted from the effects of AChE peptide on tissue cultures which are disclosed in published International Application WO 97/35962.

Neurotoxic effects have also been observed by injection of the AchE peptide into mice. The peptide is expected to act similarly in other non-human animals.

Example 4 below details further studies which have shown that attentional deficit reminiscent of Alzheimer's Disease can be produced by a single injection of biotinylated AchE peptide into the NBM of rats. As indicated above, such rats, combined with testing for attention by means of a serial reaction choice task, are now envisaged as a favoured means of testing agents for potential therapeutic utility in relation to Alzheimer's Disease.

Details for performing a serial choice reaction task for assessment of attention have previously been given above with reference to Higgs et al. European Journal of Neuroscience (2000) 12, 1781-1788 and Figure 11. The following is a list of some other behavioural tests which will be suitable for use in accordance with the invention. Most but not all of these are tests of cognitive function. Tests which relate to behaviour but not cognitive faculties are also included and may be used instead of or in addition to the tests of cognitive function such as memory.

Attention

Carli, M., Robbins, T.W., Evenden, J.L. and Everitt, B.J. (1983) Effects of lesions to ascending noradrenergic neurones on performance of a 5-choice serial reaction time task in rats; implications for theories of dorsal

noradrenergic bundle function based on selective attention and arousal. Behavioural Brain Research 9, 361-380.

Social Behaviour

Gardner, C.R. and Guy, A.P. (1984) A social interaction model of anxiety sensitive to acutely administered benzodiazepines. Drug Dev. Res. 4, 207-216.

Emotional reactivity

Gray, J.A. (1982) The neuropsychology of anxiety

Dawson, G.R. and Tricklebank M.D. (1995) Use of the elevated plus maze in the search for novel anxiolytic agents. TIPS 16, 33-36.

Morris water maze

Morris, R.G.M., Garrud, P., Rawlins, J.N.P. and O'Keefe, J. (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297, 681-683.

Radial arm maze

Olton, D.S. and Samuelson, R. J. (1976) Remembrance of places past: spatial memory in rats. Journal of Experimental Psychology: Animal Behaviour Processes 2, 97-116.

T-maze

Rawlins, J.N.P. and Olton, D.S. (1982) The septo-hippocampal system and cognitive mapping. Behavioural Brain Research 5, 331 – 358.

The examples below further illustrate the invention.

EXAMPLES

Example 1

Direct injection of AChE peptide into rat hippocampus – Histological effects

AChE peptide was injected into rat hippocampus and subsequently the brains were examined under a light microscope. The hippocampus was chosen as the most suitable brain structure in which to show effects of AChE peptide as it is very vulnerable to neurotoxins and damage from other causes such as ischaemia. Post mortem histological examination or pre mortem brain scans reveal prominent decay of the hippocampus in Alzheimer brains.

Detailed Method

Male Wistar or Dark Agouti (DA) rats were anaesthetised with Avertin and injected with 0.5 µl of 2 mM AChE peptide (DA rats received 1 µl of 1 mM) in the right hippocampus. An equivalent control injection was made in the left hippocampus. After recovery from the anaesthetic and a few days in their home cages rats were deeply anaesthetised and transcardially perfused with formalin preservative, the brains removed and prepared for microscopical examination. Sections of 50 µm were cut on a sliding microtome and stained with cresyl violet to show the nerve cells. The amount of neural damage was estimated by counting the length of hippocampal cell layers destroyed (quantified in terms of microscope graticule units).

Results

In the most symmetrically injected DA rat (to give the fairest comparison between control and AChE peptide injected sides of the hippocampus) there were 25 units of damage on the control side, 119 on the AChE peptide injected side.

In a systematic assessment of the Wistar rat brains, there

was a mean of 7.4 units of damage on the control side, 155.3 on the AChE peptide side. The difference between control and AChE peptide sides was statistically significant ($P = 0.008$).

NB. In the above example, a P value of 0.008 means the probability of this result occurring by pure chance is only 8 parts in 1,000.

These results show small areas of damage after injection of AChE peptide into the hippocampus, a crucial brain structure in memory and Alzheimer's disease.

Comparison with NMDA

A small number of injections were made into the hippocampus of DA rats with the reference neurotoxin NMDA (N-methyl D-aspartic acid) at an equivalent concentration to that of AChE peptide used above (1 µl of 1 mM). Histological examination of these brain sections showed small areas of neural damage.

Since the concentration of NMDA used to produce experimental neural destruction (as a tool in experimental psychology to examine the function of a particular brain region) is much higher (typically 68 mM) a small number of rats were injected with this larger amount, in a volume of 1 µl, in comparison with an equivalent amount of AChE peptide. Results showed that AChE peptide produced local hippocampal damage, whereas that from NMDA was more widespread.

One of the two rats given AChE peptide at this higher dose looked very sick 5 days after the surgery and was therefore deeply anaesthetised and the brain prepared for microscopy. Severe body weight loss was also noted. The other rat survived 11 days until it was finally anaesthetised, but it too lost some weight. This supported informal observations from the earlier, low dose experiment that AChE peptide treated rats might lose more weight than is normal after such surgery.

The brain of this surviving rat was prepared for microscopical examination as above. There was a small amount of (presumably mechanical) damage on the left (control) injection site along the line of the

injection needle. The right side, injected with AChE peptide, showed much more extensive damage, particularly in the overlying cortex, probably due to AChE peptide solution refluxing up the track left by the injector as it was withdrawn. The hippocampus itself showed a small localised area of cell loss. This appeared less than after injection of the reference neurotoxin NMDA, which typically extended over many consecutive brain sections.

Example 2

Effects of AChE peptide injected into the medial septum/diagonal band of Broca (S/DB) on behaviour, weight and histology

Methods

Wistar rats (175 - 200 g) were received from the suppliers (Harlan UK) and acclimated to the laboratory for three weeks before being trained on a delayed non-matching to sample (DNMS) task on an elevated wooden T-maze. In this task they are mildly food deprived and receive food pellets with enhanced flavour and nutritional content as rewards.

The DNMS task capitalises on the rat's innate tendency to alternate which cross arm of the T-maze it runs along; this derives from its normal foraging behaviour, where returning to a place from which it has recently eaten all the food is unlikely to pay dividends.

Each rat is placed at the start of the stem of the T-maze and allowed to run to retrieve a food pellet at the end of one cross arm. Access to the other cross arm is blocked. This block is then removed and the rat returned to the start. Normal rats choose to go to the opposite arm to that recently visited on this free choice part of the trial. After being allowed to consume their reward (if a correct choice was made) they are returned to their home cage and their cagemate is then tested in the same way. Typically a squad of ten rats is run in a "round robin' fashion, so up to fifteen minutes intervenes between one trial and the next. Normal rats excel at this task once initial training (chiefly to familiarise them with the new smells on the maze and the elevated position off the floor) is complete.

The rats in the present experiment were given forty trials on the T-maze, then divided into groups matched for performance (all were above 90 % correct). They were returned to unlimited food for a few days before undergoing surgery under Avertin anaesthesia.

The experimental group (n = 6 rats) received an injection of AChE peptide into the medial septum/vertical limb of the diagonal band (S/DB). Control rats (n = 6 rats) received an equal amount of the vehicle, water, in the same way, while another group (n = 5 rats) received an injection of the reference neurotoxin NMDA at an equivalent dose to that of AChE peptide (2 µl of 33 mM). Each injection was given slowly over 15 minutes through a 34 gauge stainless steel injection needle coupled by polyethylene tubing to a 10 µl Hamilton syringe. Co-ordinates used on the stereotaxic instrument (Kopf) were 0.7 mm anterior to bregma, 1.0 mm lateral to midline and 6.5 mm from skull surface at bregma. The incisor bar was set at -0.5 mm for all rats, resulting in approximately a level skull surface between the bregma and lambda sutures. The arm of the stereotaxic instrument was angled at 10 degrees to vertical to avoid damage to the sagittal sinus.

Post-operatively the rats were weighed every day. Approximately two weeks after operation they were again food restricted and tested for performance on the T-maze.

After forty trials conducted exactly as before their brain operation, the task was made more difficult by giving twenty massed trials (i.e. without the normal 10 - 15 minute gap between each individual trial). A further more difficult variant was to impose an approximately 45 second delay between sample and choice trials. The rat would be returned to its home cage and partner during the delay, and this would serve as a "forget" cue as it normally signalled the end of one trial and a waiting period before beginning the next one.

After completion of T-maze testing rats were returned to a free feeding regimen before being tested on two successive days in standard locomotor activity cages. Each cage was equipped with two

infrared beams to detect movement.

After a final weighing, rats were killed by stunning followed by decapitation. The hippocampus and cingulate/secondary motor cortex were rapidly removed and stored frozen at -80 degrees Celsius. The block of brain anterior to the hippocampus was placed in 30% sucrose formalin and left to fix for at least a week prior to sectioning on a microtome and staining with cresyl violet. Microscopical brain examination was subsequently performed as in earlier experiments.

Results

Weight and behaviour

All rats lost some weight after surgery. Whereas NMDA treated rats lost more weight than controls, they regained it quicker than the AChE peptide treated rats which were statistically significantly slower than either controls or NMDA rats to recover their weight (see Figure 2).

It was noted that one AChE peptide rat in particular seemed to display aberrant behaviour typical of hippocampally lesioned rats immediately post-operatively; it was hyperdefensive, displaying an "upright boxing" posture to both partner and experimenter.

When tested post-operatively on the T-maze, AChE peptide treated rats showed twice the deficit seen in the NMDA group on the first block of ten trials. The latter's performance subsequently returned to control levels, whereas that of the AChE peptide treated group remained low (see Figure 3).

Massing the trials had little effect on the performance of the control and NMDA groups, while that of the AChE peptide treated group continued to be poor (see Figure 4).

Imposing a delay between sample and choice produced a non -significant decrease in the NMDA group relative to control, whereas the deficit in the AChE peptide group was significantly greater than both (see Figure 5).

The ANOVA (Analysis of Variance) statistical test performed

on the locomotor activity test data showed that there were significant effects of days (i.e. activity was less on day 2 than day 1) and group (the AChE peptide group B was more active than the controls, which were not significantly different from NMDA) (see Figure 6).

The final weighing (see Figure 7) showed that all rats eventually gained weight before being anaesthetised approximately one month after the operation.

Histology

There were only small effects of NMDA on the histological appearance of the septal area. In the AChE peptide treated group, however, and especially in the two rats which were the worst performers on the T-maze, there was striking damage to not only the septal region itself but also the fornix which is the axonal pathway from the septum to the hippocampus (see Figure 9). In the worst cases these structures had virtually disappeared. This was totally unexpected given the localised damage seen after the earlier experiment with hippocampal injections, and was unlike anything normally seen after conventional neurochemical lesions.

Further experiments and replications in the same (Wistar) strain of rats and a different one (Dark Agouti) have shown that the behavioural and histological effects of the AChE peptide are replicable.

Use of the AChE peptide to model Alzheimer's disease.

These results suggest a number of ways that the AChE peptide could be used in the search for therapies for Alzheimer's disease. Typically it could be co-administered with a putative therapeutic agent and the behaviour and brains of the animals could be examined to see if the characteristic AChE peptide dysfunctions were prevented. Animal species other than rodents such as primates could be used for different or more sophisticated paradigms.

Example 3

Experiments to compare effects of AChE peptide against butyrylcholinesterase peptide (peptide C) and a scrambled version of AChE peptide

Methods

Wistar rats were anaesthetised and injected as described in Example 2, with one of the following: AChE peptide, the equivalent peptide from BuChE, a scrambled version of the AChE peptide: HSWRAEVFHKYWSM, NMDA and water as a control. The number of rats in each group was between four and five.

Brain examination was performed as described in Example 2.

Results

Significant tissue loss was observed in the AChE peptide treated rats (see Figure 9).

Example 4

Effect of biotinylated AChE peptide injected into the nucleus basalis of Meynert of rats on performance in a serial choice reaction task

Test for cognitive function

Testing for loss of cognitive function reminiscent of loss of such function associated with Alzheimer's Disease in humans was carried out using a serial choice reaction task, a preferred form of task for testing for attentional deficits, as described by Higgs et al. (Higgs,S., Deacon, R.M. J. & Rawlins, J. N. P., European Journal of Neuroscience (2000) 12, 1781-1788).

Apparatus

A rat testing cubicle with a retractable lever on the front wall was employed as illustrated in Figure 11. The back wall was concave with

three food magazines, one in each corner and one in the centre. A light was present within each corner magazine. Magazine entries were recorded by microswitches attached to panels covering the entrances. The entire apparatus was computer-controlled.

Procedure

Rats are trained to press the lever when it is presented. This results in one of three events: a light can flash either from the left magazine, or the right magazine, or there is no light. The rat will find a reward (a 45 mg food pellet) respectively in the left, right or centre magazine tray. Thus, a left response to a right flash (incorrect) suggests that the rat saw the flash but was not paying sufficient attention to where it came from. A response to the centre tray would suggest that he thought there had been no light flash.

With control rats, as the light stimulus duration is decreased from 1.0 s to 0.4 s, response accuracy has been found to decrease. This is reflected as an increase of errors to the centre tray, as if the rats had not seen a light (Higgs et al., *ibid,* Experiment 3; see also appended Figure 12)

Animal treatment

A cohort of rats were trained on the attentional task (light stimulus duration 1.0 s). When performance was stable, they were divided into three matched groups, each with seven or more rats. Under deep anaesthesia, each rat was placed in a stereotaxic frame, with the incisor bar set at -3mm to give a level head. Bilateral injections, each of 2 µl, were made into the NBM area (see Figure 13). The control group of rats received injections of water. The second group of rats received an aqueous solution of biotinylated AChE peptide and the third group received a solution of the known neurotoxin NMDA. The concentration of biotinylated AChE peptide and NMDA employed was 16.5 mM. Rats were allowed at least a week to recover from the operation.

After a period of post-operative testing, the rats were terminally anaesthetised and perfused intracardially with ice cold saline. The brain was removed and a portion of cortex (including the frontal, parietal and temporal areas, but excluding visual and cingulate cortex) was removed from each hemisphere and frozen. The remaining brain was preserved in formalin and subsequently coronally sectioned and stained with cresyl violet. The position and extent of the lesions was then determined by microscopical examination.

The frozen cortical tissue was used to determine the effectiveness of the NBM lesion. Using the method of Fonnum (J. Neurochem. (1975) 24, 407-409), the levels of the acetylcholine synthesising enzyme, choline acetyltransferase (ChAT) were measured. Since a proportion of this enzyme is due to the cholinergic innervation from the NBM, a decrease of ChAT would corroborate visualisation of a lesion at the site of injection in the NBM itself.

Results

There was a selective effect of biotinylated AChE peptide on the attentional task. The proportion of correct lever-light trials was lower (Figure 14, left panel) for rats treated with the biotinylated AChE peptide than for the controls. Thus, when a rat in the peptide-treated group pressed a lever and a light *did* come on, the rat frequently acted as if no light had been presented and defaulted to the centre tray (which would have been correct had there been no light (Figure 14, right panel). Thus treatment with biotinylated AChE peptide produced a similar effect to decreasing light stimulus duration for control rats.

Microscopical examination revealed that the peptide injections had produced small or moderate size lesions in the NBM region.

The cholinergic loss in the cortex as measured by HPLC ChAT level was 12.1% for the NMDA comparison lesions, which didn't produce a significant reduction in correct lever-light trials, as compared to 8.4% for the peptide lesion.

Conclusion

Injection of biotinylated AChE peptide into rat NBM can produce histological damage which is functionally reflected in impairment in carrying out an attentional task. This effect on attention cannot be attributed to a selective cholinergic deficit since an equivalent injection of NMDA produces a greater reduction in cortical ChAT, but does not produce the same selective effect on attention. Injection of biotinylated AChE peptide, or an active variant thereof, into rat NBM is believed to represent a novel means of more accurately mirroring brain dysfunction in brains of Alzheimer's Disease patients, in particular attentional deficit.

Although the above discussed studies are confined to rats, it can be predicted that similar attentional deficit may be produced in other non-human animals by injecting biotinylated AchE peptide, or an effective variant thereof capable of causing comparable brain lesions, into the NBM.

CLAIMS

1. A method of providing an animal model for a neurodegenerative disease which comprises introducing an effective amount of a peptide having the sequence:

AEFHRWSSYMVHWK (SEQ. ID. no.1)

or an active variant of the peptide, into one or more sites in the brain of a non-human animal whereby said peptide causes cellular degeneration and thereby impairment of a testable brain function, wherein impairment of the same brain function in a human is indicative of a neurological disorder.

2. A method as claimed in claim 1 wherein said neurological disorder is Alzheimer's Disease and said impairment is impairment of a cognitive function.

3. A method as claimed in claim 2 wherein said impairment is an attentional deficit.

4. A method as claimed in claim 2 wherein the peptide is introduced into a site in the septohippocampal system.

5. A method as claimed in claim 4, wherein the peptide is introduced at the medial septum/diagonal band of Broca (S/DB) region of the brain.

6. A method as claimed in claim 2, wherein the peptide is introduced into a site in the cortical cholinergic system.

7. A method according to claim 6, wherein the peptide is introduced into the nucleus basalis magnocellularis (NBM).

8. A method according to claim 7 wherein the peptide of SEQ. ID. no. 1 is employed in biotinylated form or a variant thereof capable of providing

functionally equivalent lesions in the NBM.

9. A method according to any one of claims 1 to 8 wherein said non-human animal is a rodent.

10. A method as claimed in any one of claims 1 to 9, which further comprises testing for said impairment.

11. An animal model for a neurodegenerative disease which is a non-human mammal treated with the peptide

AEFHRWSSYMVHWK (SEQ. ID. no 1)

or an active variant thereof in accordance with any one of claims 1 to 9.

12. An animal model according to claim 11 which is an animal model for Alzheimer's disease exhibiting attentional impairment.

13. An animal model according to claim 12 which is a rodent treated in accordance with claim 7 or claim 8.

14. A method as claimed in any one of claims 1 to 10 which further comprises administering prior to, simultaneously or after the peptide a test agent and determining whether said agent can inhibit, prevent or increase impairment of said testable brain function and/or can inhibit, prevent or increase cellular damage in the brain.

15. A method of testing an agent for biological activity in a neurodegenerative disorder which comprises administering said agent to an animal model prepared in accordance with any one of claims 1 to 10 and determining whether said agent will inhibit, prevent or increase impairment of said testable brain function and/or cause improvement or deterioration of cellular damage in the brain.

16. A method as claimed in claim 14 or claim 15 wherein said animal model is an animal model of Alzheimer's disease and said testable brain function is a cognitive function.

17. A method as claimed in claim 16 wherein impairment of attention is determined using apparatus providing a serial choice reaction task.

18. A method as claimed in claim 17 wherein said animal model is an animal model according to claim 13.

19. A test agent identified by a method according to any one of claims 14 to 18 which inhibits or prevents impairment of said testable brain function.

20. A method as claimed in any one of claims 14 to 18 wherein a test agent is selected which is a compound capable of inhibiting or preventing impairment of said testable brain function and which further comprises synthesising said compound.

21. A method as claimed in any one of claims 14, 15 and 20 which further comprises incorporating said agent into a pharmaceutical composition together with a pharmaceutically acceptable carrier or diluent.

22. A method as claimed in claim 21 wherein said agent is a compound and said pharmaceutical composition is suitable for delivery of said compound across the blood-brain barrier.

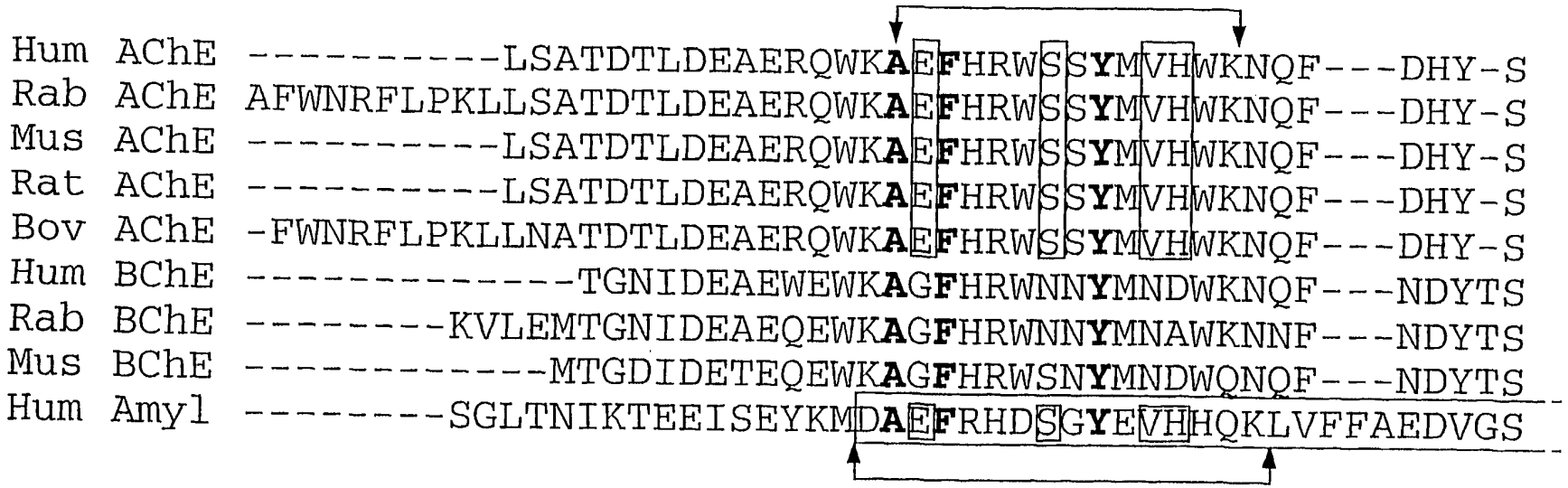
23. A pharmaceutical composition comprising a test agent as claimed in claim 19 together with a pharmaceutically acceptable carrier or diluent.

24. Use of an agent selected by a method as claimed in any one of claims 14 to 18 which inhibits or prevents impairment of said tested brain function for the manufacture of a medicament for use in the treatment of a

neurological disorder.

25. Use of an agent selected by a method as claimed in any one of claims 16 to 18 which inhibits or prevents impairment of a tested cognitive function of relevance to Alzheimer's disease for use in the manufacture of a medicament for use in the treatment of said disease.

Fig.1.



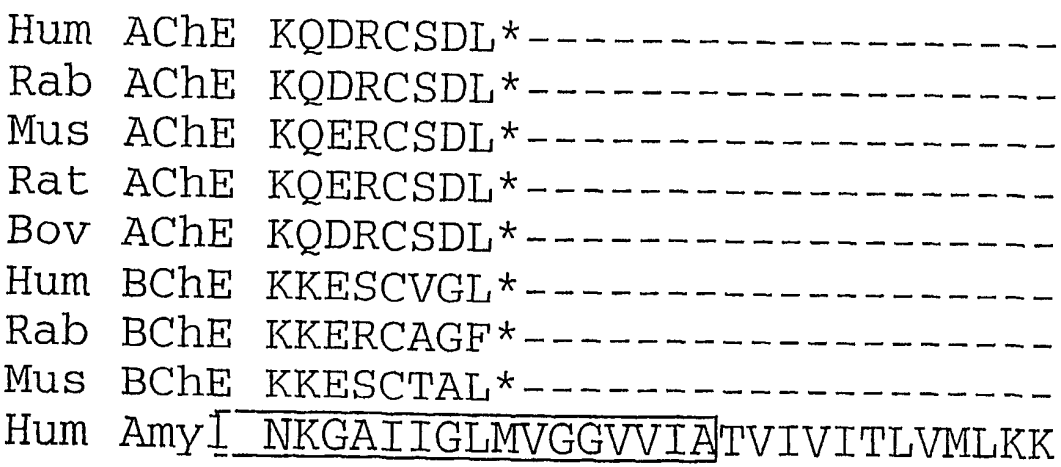


Fig.2.

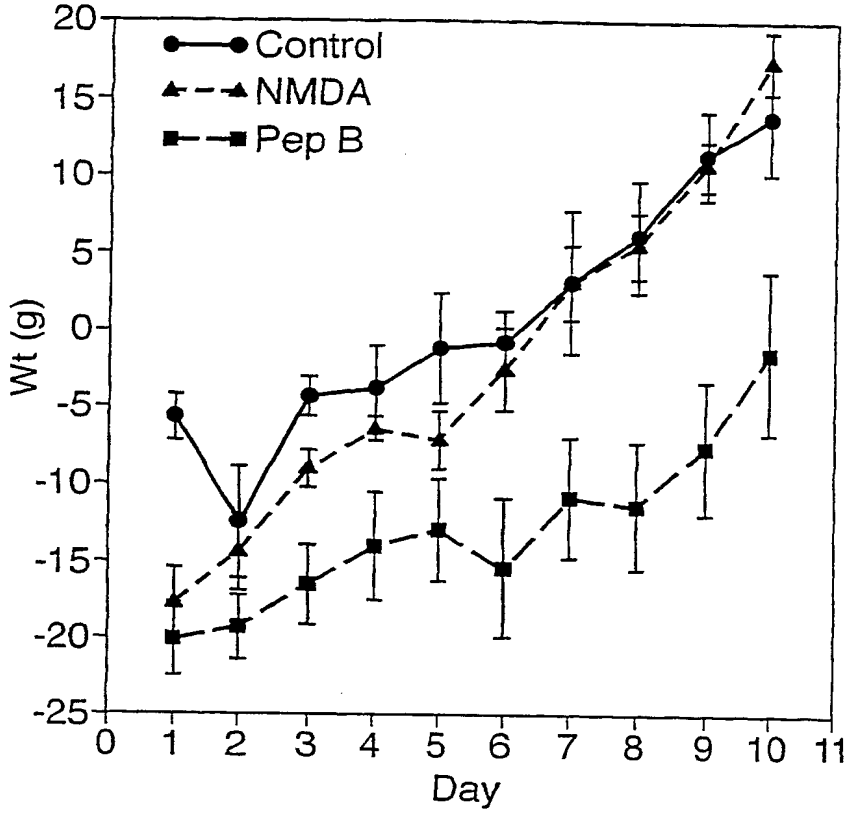

Control
NMDA
Pep B
Wt (g)
20
15
10
5
0
-5
-10
-15
-20
-25
0 1 2 3 4 5 6 7 8 9 10 11
Day

Fig.3.

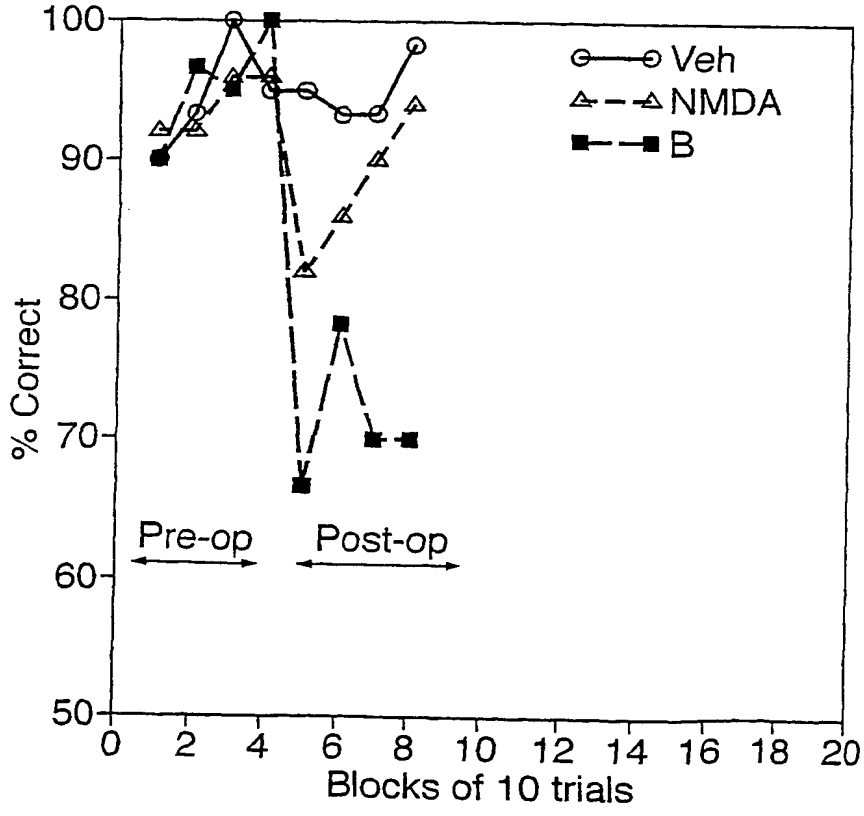

Veh
NMDA
B
% Correct
100
90
80
70
60
50
Pre-op
Post-op
0 2 4 6 8 10 12 14 16 18 20
Blocks of 10 trials

Fig.4.

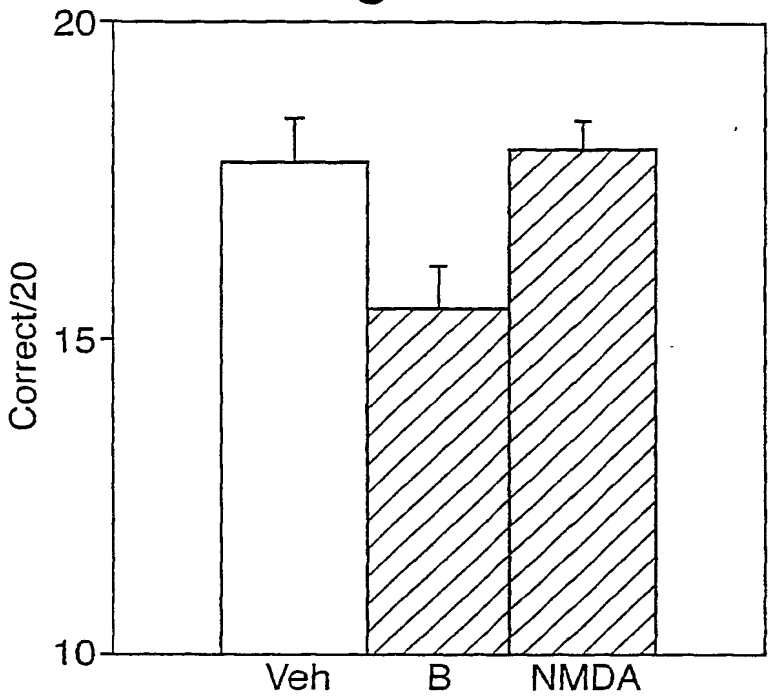
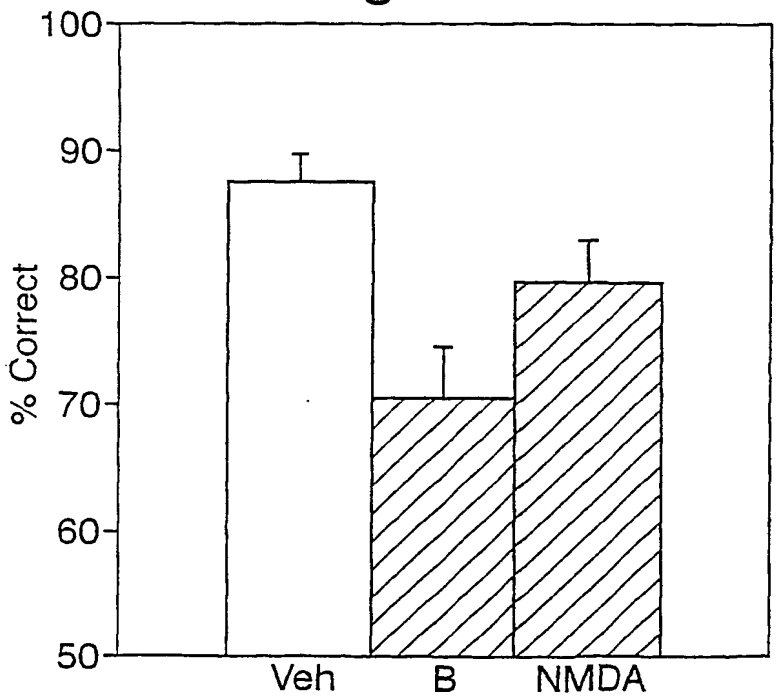

20
15
10
Correct/20
Veh
B
NMDA

Fig.5.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90
80
70
60
50
% Correct
Veh
B
NMDA

Fig.6.

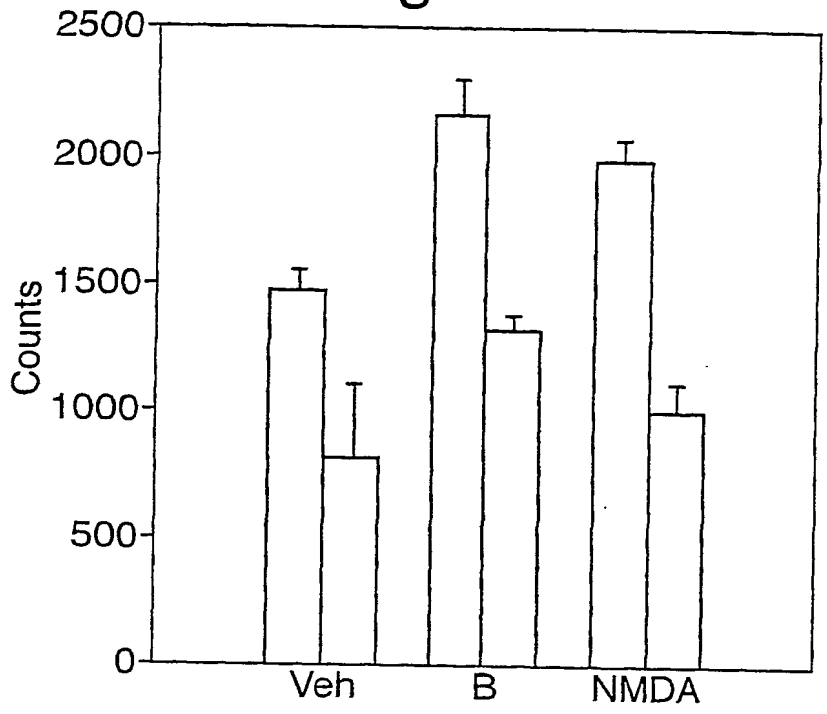
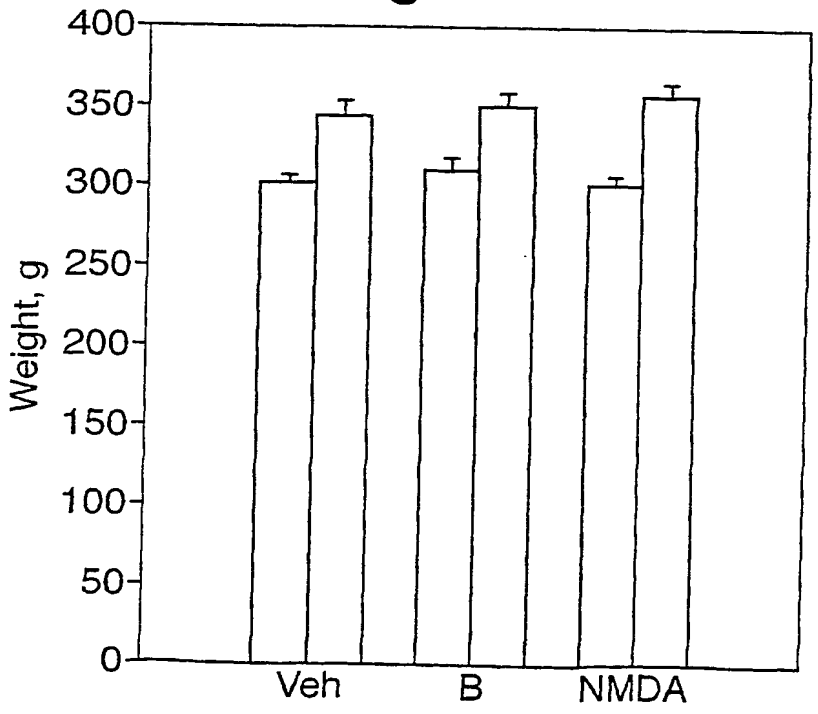

2500
2000
1500
1000
500
0
Counts
Veh
B
NMDA

Fig.7.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350
300
250
200
150
100
50
0
Weight, g
Veh
B
NMDA

Fig.8.

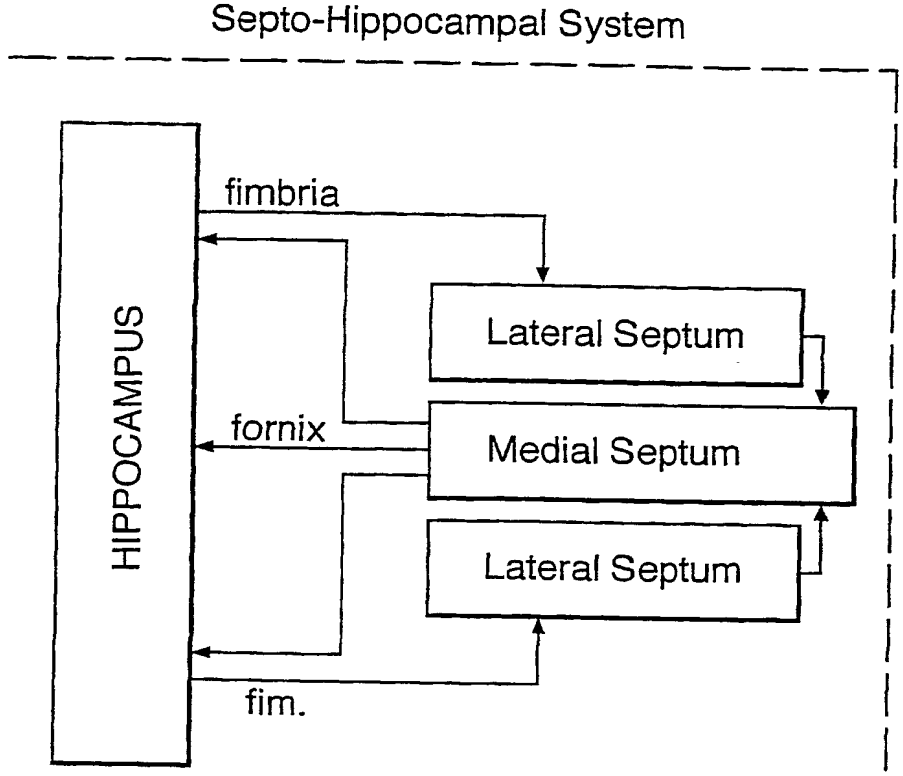
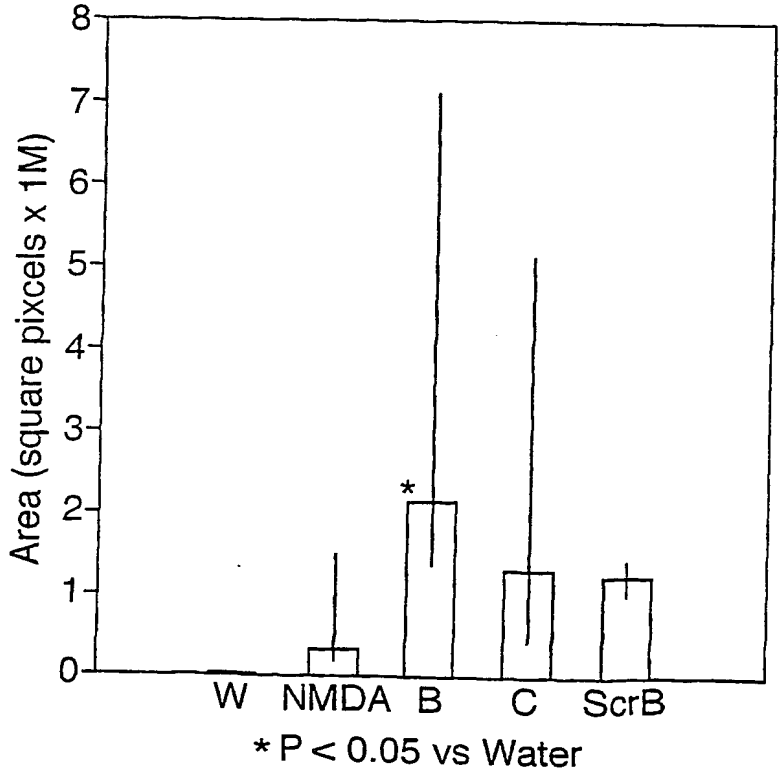

Septo-Hippocampal System
HIPPOCAMPUS
fimbria
fornix
fim.
Lateral Septum
Medial Septum
Lateral Septum

Fig.9.


Area (square pixcels x 1M)
0
1
2
3
4
5
6
7
8
*
W
NMDA
B
C
ScrB
* P < 0.05 vs Water

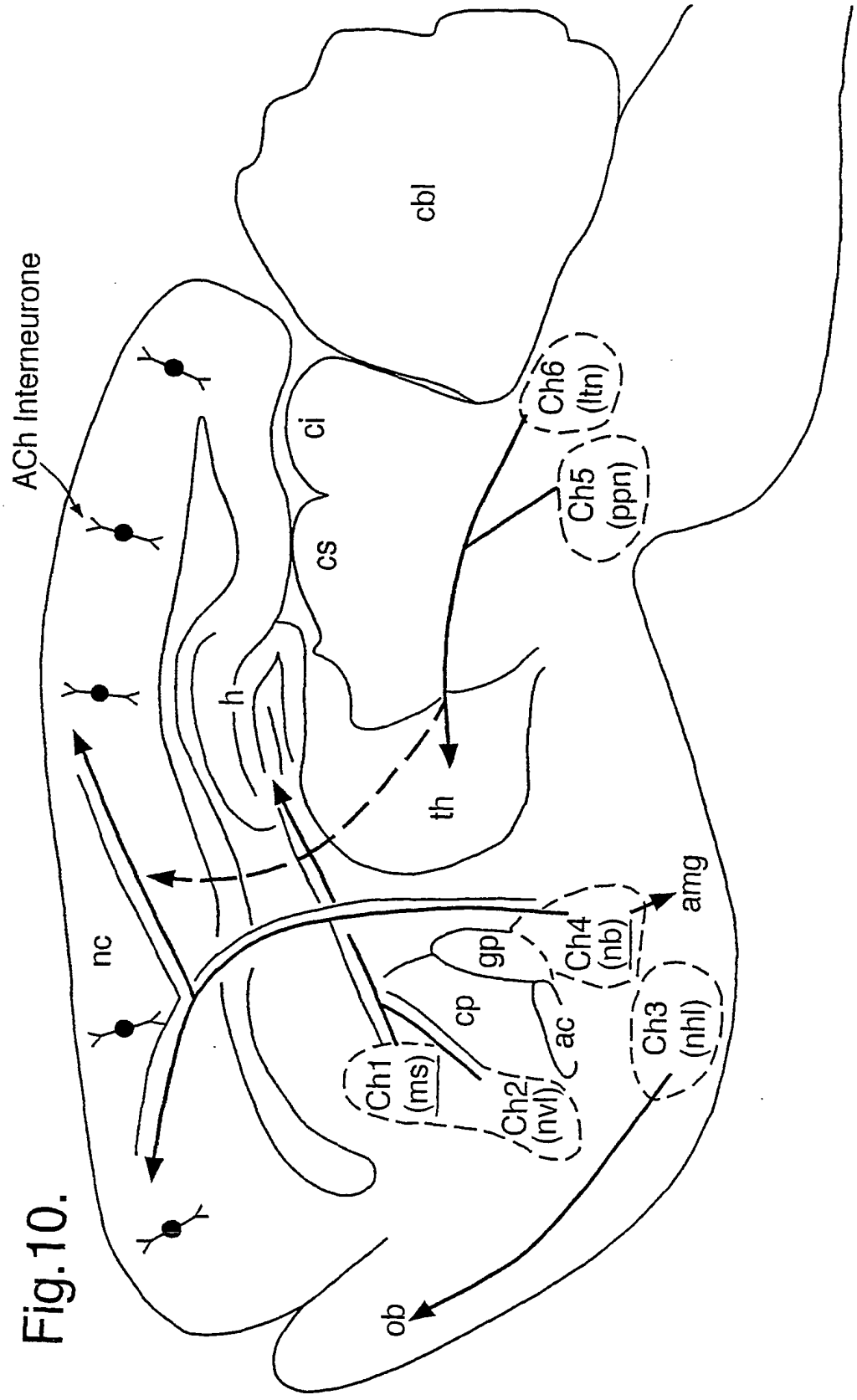

ACh Interneurone
cbl
ci
cs
h
th
Ch6 (ltn)
Ch5 (ppn)
nc
gp
Ch4 (nb)
amg
cp
ac
Ch3 (nhl)
Ch1 (ms)
Ch2 (nvl)
ob

Fig.10.

Fig.11.

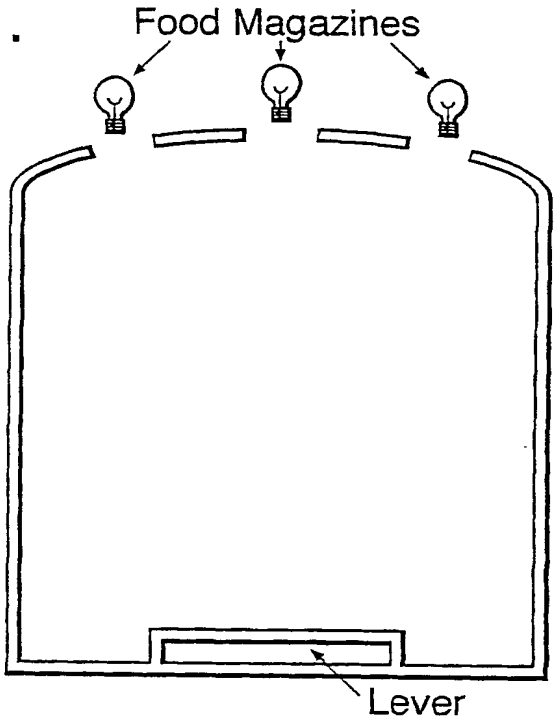


Fig.13.

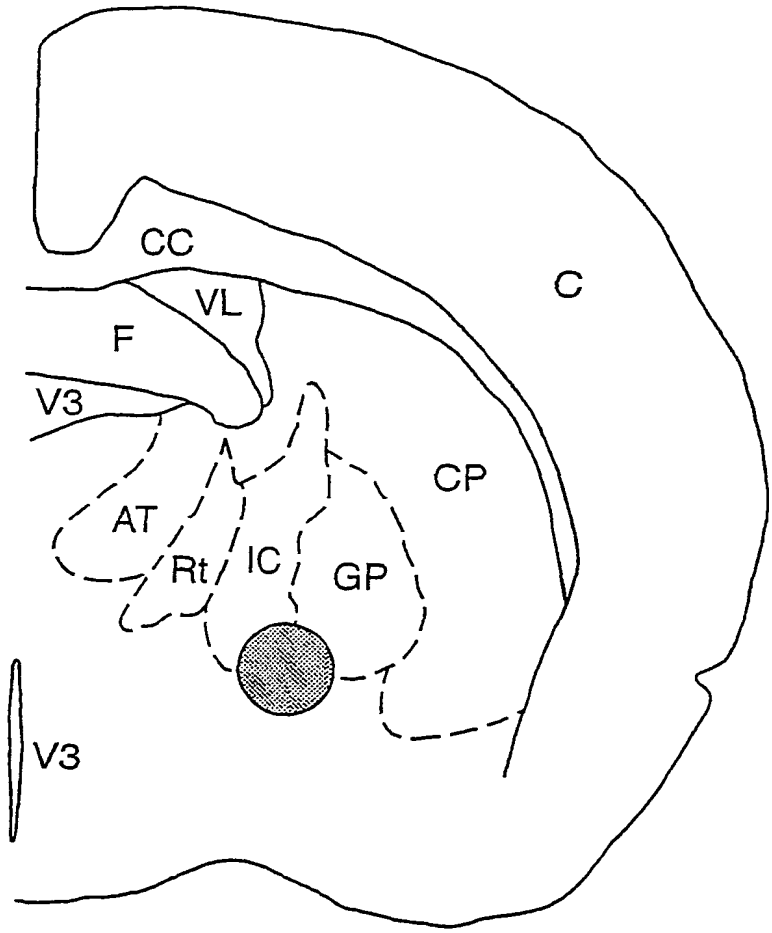


Fig.12.

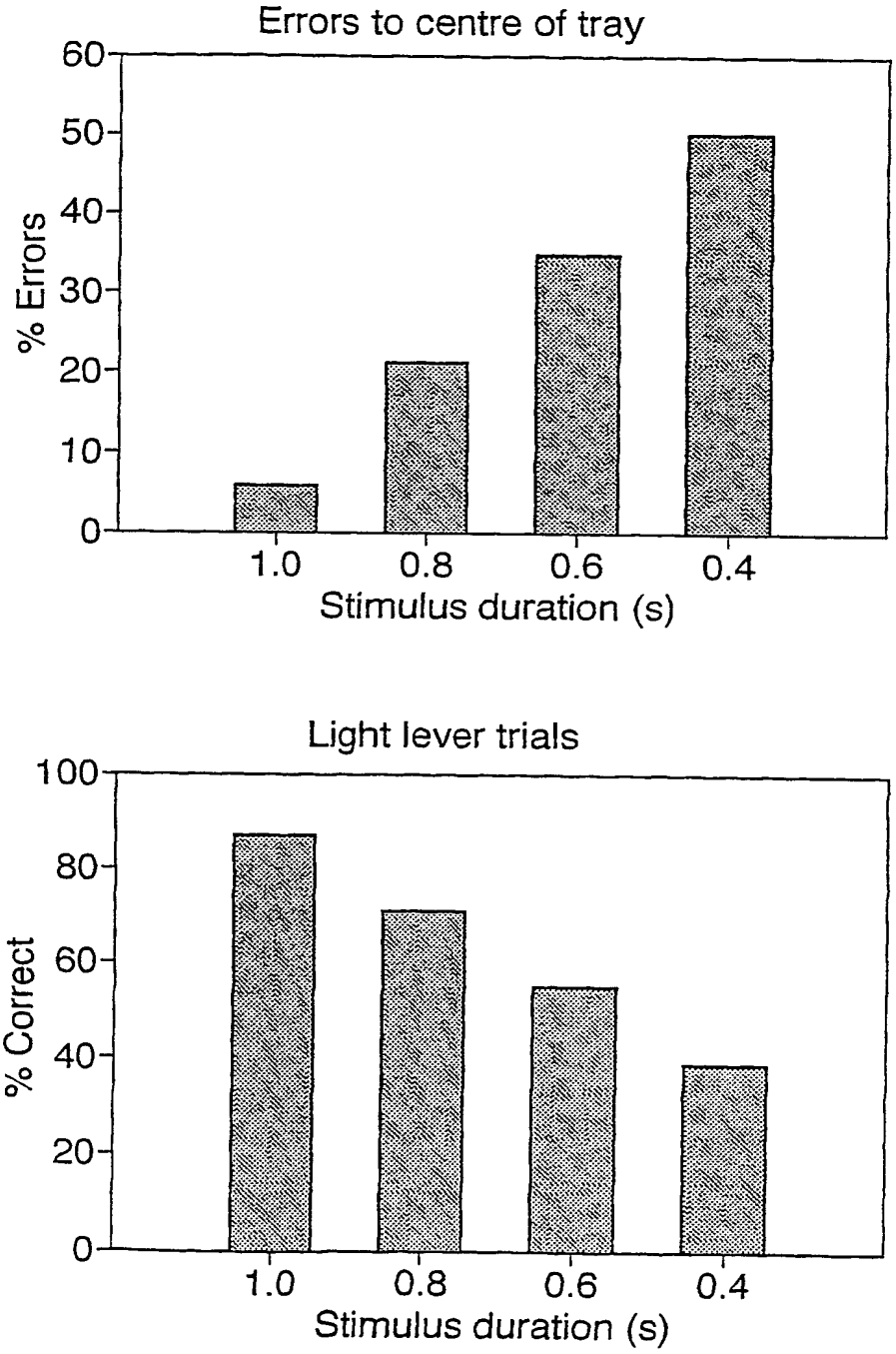
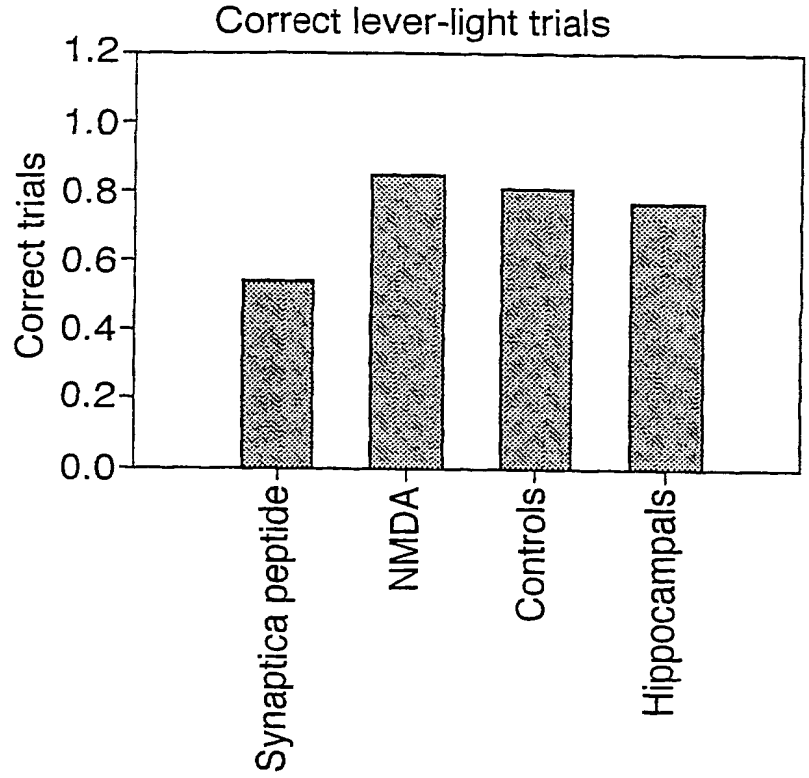
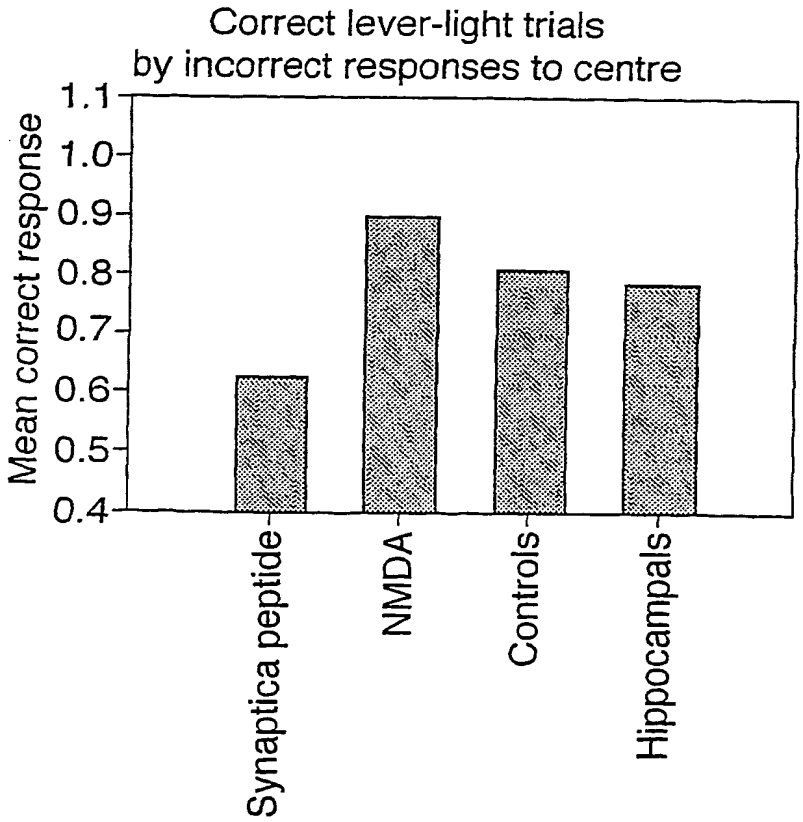

Errors to centre of tray
% Errors
60
50
40
30
20
10
0
1.0
0.8
0.6
0.4
Stimulus duration (s)
Light lever trials
% Correct
100
80
60
40
20
0
1.0
0.8
0.6
0.4
Stimulus duration (s)

Fig.14.


Correct lever-light trials
Correct trials
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Synaptica peptide
NMDA
Controls
Hippocampals


Correct lever-light trials
by incorrect responses to centre
Mean correct response
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Synaptica peptide
NMDA
Controls
Hippocampals

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04991

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01K67/027 C07K7/04 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35962 A (VAUX DAVID JOHN TALBUTT ;ISIS INNOVATION (GB); GREENFIELD SUSAN AD) 2 October 1997 (1997-10-02) cited in the application claims 5,10	19,23-25
Y	page 4, last paragraph -page 5	1-25
Y	WO 95 35366 A (UNIV PENNSYLVANIA ;TROJANOWSKI JOHN Q (US); LEE VIRGINIA M Y (US);) 28 December 1995 (1995-12-28) claim 37	1-25

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2001	21/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Deffner, C-A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/04991

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9735962 A	02-10-1997	EP 0896615 A	17-02-1999
		JP 2000509967 T	08-08-2000
WO 9535366 A	28-12-1995	US 5849988 A	15-12-1998